Tissue engineering for novel female infertility treatments

Studies on small and large animal models

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Front cover: The principle of decellularization and recellularization.

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Dedicated to the two women integral in my life:
my mom for teaching me that even if I lose everything, I can build everything back up with my knowledge
&
my wife for putting up with my shenanigans and for supporting me throughout my doctoral studies

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“Live as if you were to die tomorrow. Learn as if you were to live forever.”
- Mohandas Karamchand “Mahatma” Gandhi

ABSTRACT

Introduction: As with any transplantation (Tx) procedure, uterus Tx is associated with risky donor surgery and adverse side-effects from immunosuppression. With the aim to bypass these risks, this thesis investigated uterus tissue engineering strategies and the potential to develop a patient-specific uterus graft to replace the need for donor surgery and immunosuppression. A translational approach for uterus scaffold production through a process called decellularization (DC) is addressed using the rat and the sheep animal model. The immunological events following engraftment of rat uterus scaffolds was also evaluated. The thesis also assessed cellular reconstruction techniques and perfusion bioreactor protocols that can be useful to recellularize whole sheep uterus scaffolds for future uterus Tx studies.

Methods: The immune response towards three different rat uterus scaffold types were evaluated after transplantation by quantifying infiltrating leucocytes and the expression of pro-inflammatory cytokines. Additionally, three novel whole sheep uterus scaffolds were produced by DC and the scaffold composition, bioactivity, mechanical strength and ability to support seeded stem cells were analyzed. Technique optimization for a perfusion bioreactor was also conducted using normal sheep uterus and a specialized perfusion medium.

Results and conclusions: In Paper I, we deciphered DC protocol-dependent differences in the immune response following engraftment. A mild, yet effective DC protocol resulted in an immune-inert scaffold type. In Paper II-III, we developed three promising extracellular matrix-derived bioactive sheep uterus scaffolds that after an enzymatic pre-conditioning were able to support wide-spread cell attachment and migration during recellularization. In Paper IV, we were able to maintain normal sheep uterus ex-vivo for 48 hours using a custom made culture medium and a perfusion bioreactor. These parameters should facilitate future whole sheep uterus tissue engineering experiments so that a patient-specific tissue engineered uterus can be made to replace a donor in a uterus Tx setting.

SAMMANFATTNING PÅ SVENSKA

Introduktion: I likhet med andra organtransplantationer är livmoderstransplantation förknippat med risker under donationskirurgin samt med biverkningar från den immunosuppressiva behandlingen. Med avsikt att försöka kringgå dessa risker undersöker denna avhandling möjliga strategier som kan leda till framställningen av en konstgjord livmoder som är patient-specifikt och som består av ett biomaterial som fyllts med patientens egna stamceller. På ett translationellt forskningssätt beskriver denna avhandling hur ett livmodersbiomaterial kan framställas för djurstudier på råtta och får. Biomaterialens förmåga att undvika en immunförsvarsreaktion efter transplantation undersöks också. Dessutom så utvärderas olika stamcellsappliceringsmetoder och olika perfusionsbioreaktorer som kan bli gynnsamma för framtida transplantationsstudier.

Metoder: Immunreaktionen utvärderades genom kvantifiering av infiltrerade immunceller efter att tre olika råttlivmodersbiomaterial transplanterats. Genuttrycksanalyser på proinflammatoriska cytokiner gjordes också. Dessutom framställdes tre nya fårlivmodersbiomaterial. Dess biologiska sammansättning och egenskaper analyserades i detalj, inklusive dess förmåga att främja stamcellstillväxt. Dessutom utvärderades ett speciellt framtaget perfusionsmedium för att hålla en fårlivmoder vid liv i en bioreaktor.

Resultat och slutsatser: I Delarbete I så upptäcktes biomaterials-specifika skillnader på aktiveringen av immunförsvaret efter transplantation. Ett milt men effektivt framställningsprotokoll producerade biomaterial som tolererades av värddjuret. I Delarbete II-III så tillverkades tre lovande fårlivmodersbiomaterial som efter en enzymbehandling kunde stimulera en mycket effektiv stamcellsuppbyggnad. I Delarbete IV så visades att en fårlivmoder kunde hållas vid liv i 48 timmar i en bioreaktor med hjälp av ett eget-utvecklat perfusionsmedium. Dessa bioreaktorparametrar gynnar fortsatta försök med mål att framställa en komplett konstgjord livmoder som skulle kunna ersätta en donator vid en livmoderstransplantation.

LIST OF ARTICLES

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Padma AM**, Alshaikh AB, Song MJ, Akouri R, Oltean M, Brännström M, Hellström M. Decellularization protocol-dependent DAMPs in rat uterus scaffolds differentially activate the immune response after transplantation. J Tissue Eng Regen Med. Under revision.

II. Tiemann TT, **Padma AM**, Sehic E, Backdähl H, Oltean M, Song MJ, Brännström M, Hellström M. Towards uterus tissue engineering: a comparative study of sheep uterus decellularisation. Mol Hum Reprod. 2020;26(3):167-78.

III. **Padma AM**, Carrière L, Krokström-Karlsson F, Sehic E, Bandstein S, Tiemann TT, Olten M, Song MJ, Brännström M, Hellström M. Towards a bioengineered uterus: bioactive sheep uterus scaffolds are effectively recellularized by enzymatic preconditioning. NPJ Regenerative Medicine. Under revision.

IV. **Padma AM**, Truong M, Jar-Allah T, Song MJ, Oltean M, Brännström M, Hellström M. The development of an extended normothermic ex vivo reperfusion model of the sheep uterus to evaluate organ quality after cold ischemia in relation to uterus transplantation. Acta Obstet Gynecol Scand. 2019;98(9):1127-38.

LIST OF CO-AUTHORSHIPS

These are articles from my time as a doctoral student where I was a co-author.

I. Kuna VK*, **Padma AM***, Håkansson J, Nygren J, Sjöback R, Petronis S, Sumitran-Holgersson S. Significantly accelerated wound healing of full-thickness skin using a novel composite gel of porcine acellular dermal matrix and human peripheral blood cells. Cell Transplant. 2017;26(2):293-307.

II. **Padma AM**, Tiemann TT, Alshaikh AB, Akouri R, Song MJ, Hellström M. Protocols for Rat Uterus Isolation and Decellularization: Applications for uterus tissue engineering and 3D cell culturing. Methods Mol Biol. 2018;1577:161-75.

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IV. Simsa R, **Padma AM**, Heher P, Hellström M, Teuschl A, Jenndahl L, Bergh N, Fögelstrand P. Systematic in vitro comparison of decellularization protocols for blood vessels. PLoS One. 2018;13(12):e0209269.

V. Alshaikh AB, **Padma AM**, Dehlin M, Akouri R, Song MJ, Brännström M, Hellström M. Decellularization of the mouse ovary: comparison of different scaffold generation protocols for future ovarian bioengineering. J Ovarian Res. 2019;12(1):58.

VI. Søfteland JM, Casselbrant A, Biglarnia AR, Linders J, Hellström M, Pesce A, **Padma AM**, Jiga LP, Hoinoiu B, Ionac M, Oltean M. Intestinal preservation injury: a comparison between rat, porcine and human intestines. Int J Mol Sci. 2019;20(13).

VII. Alshaikh AB, **Padma AM**, Dehlin M, Akouri R, Song MJ, Brännström M, Hellström M. Decellularization and recellularization of the ovary for bioengineering applications; studies in the mouse. Reprod Biol Endocrinol. 2020;18(1):75.

VIII. Søfteland JM*, Bagge J*, **Padma AM**, Hellström M, Wang Y, Zhu C, Oltean M. Luminal polyethylene glycol solution delays the onset of preservation injury in the human intestine. Am J Transplant. 2020

CHAPTERS

ABBREVIATIONS

AB – alcian blue

ART – assisted reproductive technologies

AUFI – absolute uterine factor infertility

CAM – chorioallantoic membrane

CD – cluster of differentiation

DAMPs – damage associated molecular patterns

DAPI – 4′,6-diamidino-2-phenylindole

DC – decellularization

ddPCR – droplet digital polymerase chain reaction

dMIQE – minimum information for publication of quantitative digital PCR experiments

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DNase –deoxyribonuclease

DPBS – Dulbecco's phosphate buffered saline

DRG – dorsal root ganglion

dUTDs – decellularized uterine tissue discs

ECM – extracellular matrix

EDD – embryo development day

GAGs – sulfated glycosaminoglycans

GFs – growth factors

h	– hours
H&E	– hematoxylin and eosin
IGL-1	– Institute George Lopez-1
IL	– interleukin
IVF	– in-vitro fertilization
MHC	– major histocompatibility factor
MMP	– matrix metalloproteinase
MSCs	– mesenchymal stem cells
MT	– Masson’s trichrome
MTT	– 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NK	– natural killer cells
P	– protocol
PBS	– phosphate buffered saline
pH	– potential of hydrogen
RC	– recellularization
RNA	– ribonucleic acid
SD	– Sprague Dawley
SF-SCs	– sheep fetal bone marrow stem cells
SDC	– sodium deoxycholate
SDS	– sodium dodecyl sulfate

TE – tissue engineering

TW – transwell

UTx – uterus transplantation

VVG – Verhoeff van Geison

WHO – world health organization

INTRODUCTION

Becoming a parent is one of the most prevalent and awaited desires in adulthood that unites all ethnic, cultural, linguistic and economic groups. However, the dream of having a child does not come true for some couples, which can cause substantial negative psychological effects for the affected persons.[1] Infertility is a growing public health concern in most parts of the world. While some infertile couples become parents with the aid of assisted reproductive technologies (ART) and others by adoption, there are still a significant number of couples who remain childless in spite of their strong wish for parenthood.

Nowadays, tissue engineering (TE) of organs is not exclusively prescribed for life-threatening conditions such as diseases of the heart, lung, liver, kidney or bone, but has now been explored as treatment options for quality of life enhancing measures, e.g. the hand or the uterus. Although clinical trials of tissue engineered grafts have been performed, it is still considered an experimental procedure. Progress is yet to be made in optimizing protocols, addressing safety & ethical concerns before advancing to future clinical trials.

Infertility

Infertility, according to the World Health Organization's (WHO) International Classification of Diseases 11th revision is described as "A disease of the reproductive system defined by the failure to achieve a clinical pregnancy after twelve months or more of regular unprotected sexual intercourse".[2,3] For women below 29 years of age, 79% of the pregnancies occur during the first six menstrual cycles of unprotected intercourse while for women of 29 years of age or above, 74% become pregnant.[4] Overall, infertility affects between 10% and 15% of couples of reproductive age worldwide,[5] which totals the amount to affect more than 72.4 million couples.[6] There are two main circumstances leading to infertility: (i) primary infertility caused by the absence of conception and (ii) secondary infertility which affect couples that have had earlier

pregnancies but are unable to get pregnant a second time. Both of these infertility causes can be further divided into: “female factor”, “male factor”, “combined factor”, “unexplained infertility” and “other causes” which correspond to 30%, 30%, 10%, 25% and 5% respectively. In spite of this fact, it is challenging to precisely establish the singular explanation for these aspects.[7,8] The causes for male factor infertility are usually due to poor semen quantity and/or quality, or prevention of ejaculation due to an obstruction of the seminal duct. It was also observed that sperm quality in males have declined by up to 50% in the last 50 years in the industrial world.[9] Regarding female factor infertility, the majority of the cases can be attributed to ovulatory dysfunctions, premenstrual syndrome, abnormal bleeding or atypical cycle length as well as several uterine factors.[10] The WHO has pinpointed frequent causes for female infertility (excluding ovarian factors) as tubal abnormalities (26%), hyperprolactinemia (4%), endometriosis (4%) and genital tract diseases (4%) that e.g. negatively affect implantation.[3,11]

Uterine factor infertility

Absolute uterine factor infertility (AUFI) is a condition where women cannot become pregnant due to an absent or a non-functioning uterus. No current standard ART may cure this infertility condition. Overall, the estimated amount of women with a uterine factor infertility is around 3-5% of the total women suffering from infertility[12] which adds up to around 4 500 fertile aged women just in the Nordic countries.[13]

Of all the uterine factor infertility conditions, a majority are caused by an acquired injury to the uterus. Myoma for example, is a common benign pathology that affects 21% to 26% of all women. More specifically, around 10% of women aged 33-40 are affected, but the condition becomes more prevalent with increasing age.[14] The cause-specific infertility among women affected with myoma is around 40%.[15] The current treatment options for this is either corrective surgery

(myomectomy) for women who are yet to have a family, or hysterectomy for women who already had children.[15]

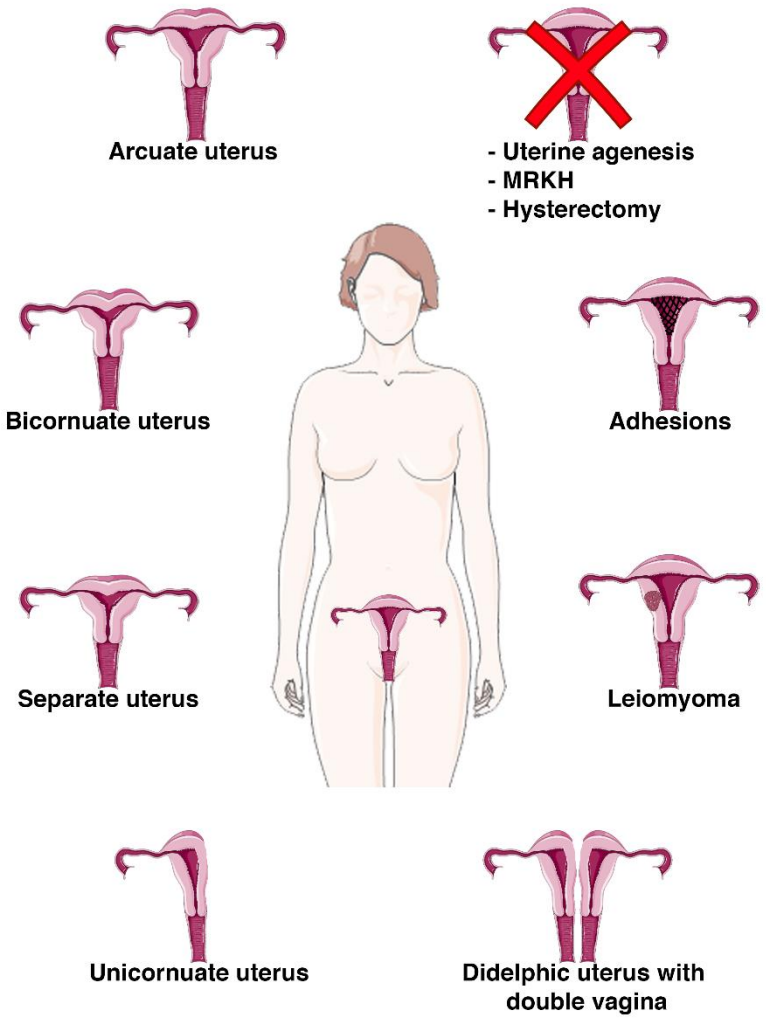

Figure 1. A diagrammatic illustration types of uterine factor infertility.

Another reason for AUFI are intrauterine adhesions (Asherman's syndrome) that lead to inability to achieve pregnancy or repetitive loss of pregnancy. These luminal adhesions are often the secondary consequence from primary intrauterine infections, genital tuberculosis or from previous surgical abortions. Untreated intrauterine adhesions can lead to an increase in infertility rates classified as AUFI.[16] Uterine

adhesions can be treated by hysteroscopic lysis of the adhesions, and in most cases, the shape and size of the uterine cavity can be restored. Nonetheless, close to two-thirds of women with serious adhesion problems do not have their fertility restored even after surgery.[17]

Sudden peripartal infertility is also a condition of AUFI. This is caused by a hysterectomy that was performed immediately or within 24 hours (h) after delivery as a lifesaving treatment for the mother due to e.g. uterine rupture, atonic bleeding or an abnormally invasive placentation. This is a condition that affects 3-4 per 10 000 women in the Nordic countries.[18]

Another cause of AUFI is cervical cancer, which is the second most common type of cancer that affects women worldwide.[19] 50% of the diagnosed women with cervical cancer are under 40 years of age,[20] and a significant number of patients are under 30.[21] There is an estimate that around half of the women who are diagnosed with cervical cancer that are below 40 years of age undergo fertility-sparring surgical interventions by the trachelectomy procedure, where the uterus is exempted during surgery while the cervix is resected.[22] The remaining women who are not eligible for the abovementioned treatment usually undergo hysterectomy, causing AUFI.[23]

Another cause of AUFI is due to congenital malformations that represents around 7% of women with AUFI.[24] This condition causes uterus abnormalities as a result of a failed absorption of the partition between the fused Müllerian ducts or a developmental abnormality that resulted in a bicornuate uterus (Figure 1). The abovementioned two syndromes could be associated with almost normal fertility or cured by surgery. Other types of congenital uterus abnormalities includes didelphic uterus or unicornuation. These cannot be cured by surgery and represents about 20% of the total cases of congenital uterus abnormalities.[25] However, there is also a less common but more severe abnormality classified as the Mayer-Rokitansky-Küster-Hauser syndrome which affects around 1 in 4500 women. These women have a complete absence of a uterus from birth, or may only have an under-developed uterus.[26]

Treatments for infertility

Treatments for male infertility

For men, there are a few treatments that alleviate some types of infertility. The most common problems are reduced sperm quality and issues with sperm motility. These problems can in most cases be overcome by intracytoplasmic sperm injection.[27] Other examples of fertility treatments may include testosterone supplementation to men who have low androgen levels,[28] or other medications to prevent erectile dysfunction caused by hypertension/angina pectoris.[29] Surgery is a solution for men who suffer from a blockage in vas deferens.[30] There is also a method to isolate sperm directly from the testicular tissue using microdissection testicular sperm extraction/aspiration that has resulted in successful outcomes in the clinic.[31] More recent advancements, but so far only in animal models, have shown that it is possible to produce artificial male (and female) gametes from induced pluripotent stem cells which also led to successful livebirths of animal offspring. Although these techniques are still in the experimental phase, this strategy may reach the clinic in the future.[32]

Treatments for female infertility

There are many treatment options for female infertility. Women who suffer from a mild subfertility with ovulation disorders may be treated with hormone supplements to improve ovarian stimulation.[33] Surgery is an option for women diagnosed with endometriosis, adenomyosis, uterine adhesions or blocked Fallopian tubes.[33,34]

The most significant advancements in infertility treatments throughout the years are the ART. Intrauterine insemination is one of the oldest method where the world's first documented procedure was performed in the UK in 1884. This method involves the placement of semen into the uterus of an ovulating woman. Although controversial in its early days, this strategy is now widely used to help couples conceive.[35] In-vitro fertilization (IVF) was a revolutionary treatment that was developed

and successfully performed by Robert Edwards in the UK in 1978 for which he was awarded the Nobel Prize in Physiology and Medicine in 2010. This method involves the aspiration of mature oocytes from follicles of the ovaries, and fertilizing it in-vitro using semen. The fertilized oocytes are then normally cultured for 3-5 days in-vitro prior to embryo transfer into the uterine cavity where it ultimately implants and continues to develop into a normal fetus. This method may benefit both infertile men and women as it overcomes problems related to reduced sperm quality/motility, and women with ovulatory problems or fallopian tube obstruction, including solving some unexplained infertility conditions.[36]

With all these advancements being revolutionary, there are still some women who cannot become fertile. These women may suffer from uterus-related infertility conditions such as AUFI, or have some type of unexplained infertility where none of the abovementioned therapies help.

Uterus transplantation

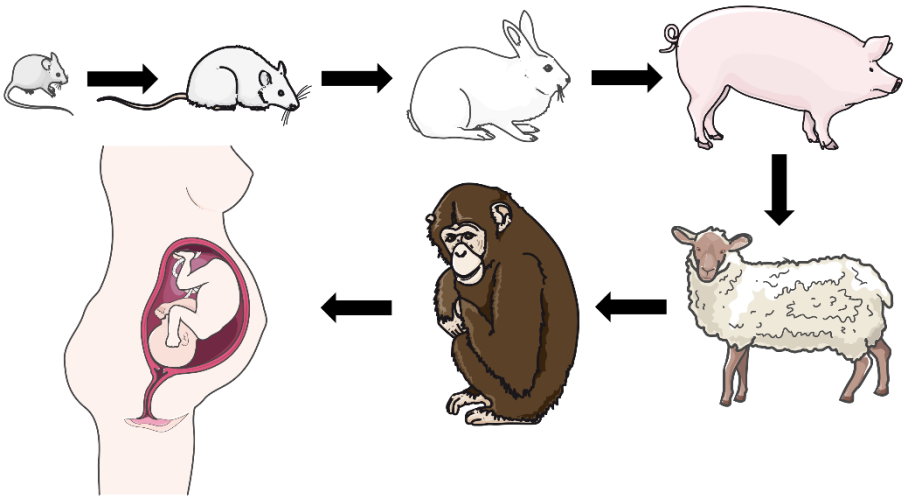

Figure 2. Translational process of uterus transplantation from the mouse, rat, rabbit, pig, sheep, non-human primates and finally, the human.

Early preclinical uterus transplantation studies

As some infertility causes (e.g. AUFI) could not be overcome by other available approaches, uterus transplantation (UTx) has been explored as a means to treat female infertility. Initial experiments on UTx was performed in 1927 on dogs where avascular grafts were placed in the omentum leading to a reasonable success based on the uteri still being viable after eight months.[37] This study was followed up in 1960s by a study where the UTx was conducted with vascular anastomoses in 14 dogs. Five dogs survived the transplant procedure after the administration of azathioprine as immunosuppression.[38] Around the same time, there was also a study that reported successful pregnancy after vascular anastomosed autologous UTx procedures in eighteen dogs that resulted in the delivery of two litters with three and nine pups, respectively.[39] In the 1970s, non-vascular anastomosed UTx in the omentum was performed in non-human primates (macaque) in autogenic and allogenic conditions. Neo-angiogenesis was observed, followed by full rejection at day 14 in the allografted group.[40]

More recent small animal models of UTx

The breakthrough of IVF in the late 1970s had lead to a decreased interest for UTx. However, in the early 2000s, with modern improvements in the fields of surgical techniques and immunosuppressive therapies, Prof. Mats Brännström initiated a research program on UTx using vascular anastomosis protocols in experimental animals. Initially, the uterus was explanted and heterotopically transplanted into syngeneic mice to evaluate surgery procedures in rodent models[41] with successful pregnancies reported shortly after.[42] Additional studies evaluated the function and viability of the uterus after engraftment following different cold ischemia protocols where the authors showed that the rodent uterus can tolerate cold ischemia times of up to 24h and still be functional.[43]

The initial rat UTx model was first reported in 2008.[44] This was a heterotopic UTx model where the syngeneic graft was anastomosed to the aorta and vena cava while the native uterus was left untouched (Figure 3). This was followed up by an orthotopic UTx that resulted in successful pregnancy after spontaneous mating.[45] This was followed by the first pregnancy and live offspring in an allogeneic model using tacrolimus as immunosuppression. The pregnancy went to term and the rat pups developed normally.[46] The effect of warm ischemia was also studied where longer warm ischemia times more than 4h detrimentally affected the viability of the uterus after transplantation.[47]

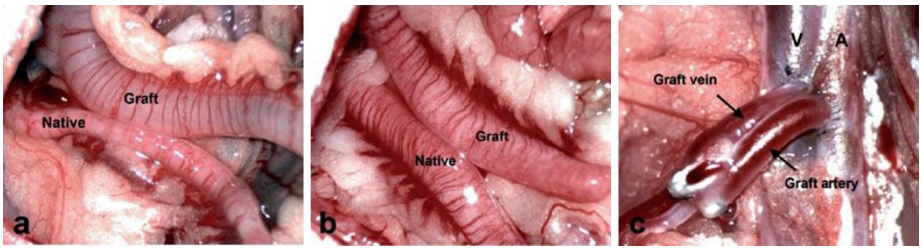

Figure 3. Figure used with permission from the publisher and authors.[44] Photographs of the grafted uterus transplanted heterotopically on a) day 1, b) day 21 and c) end to side anastomosed blood vessels in the rat.

Large animal models

These successful UTx protocols for rodents were then translated and evaluated on larger animal models. The first autologous UTx reported on pigs was conducted in 2005 and showed that there were problems after engraftment due to a gradual formation of vascular thrombosis.[48] Another study on autologous pig UTx was performed where the uterus had been preserved with Ringer's acetate solution during the cold ischemia time before being and re-transplanted. However, The results from this study were slightly better than the earlier studies on the pig, with successful blood reperfusion seen in 4 out of 19 animals.[49] An allogeneic UTx study was then performed in the smaller mini-pig model with the administration of tacrolimus and cyclosporine as immunosuppression. This study showed that the grafted uterus was viable in half of the transplanted pigs one year after UTx.[50]

The porcine animal model is often used in development and training of surgery, and is considered the best large preclinical animal model after non-human primates. However, for female infertility studies, the sheep is considered the best animal model since the size of the uterus and the vascular anatomy are more comparable to the human uterus. The first autologous sheep UTx was performed in 2008 where successful blood reperfusion was observed in 5 out of 7 transplanted animals.[51] The surgical technique was then modified where the uterus was transplanted with one uterus horn along with the ovary. The outcome of the study had favorable outcomes with a 50% animal survival rate, and 60% of the surviving animals became pregnant after natural mating.[52] Prof. Brännström's team continued to look into various aspects of UTx protocols and also investigated the effects of warm and cold ischemia injury-related damage to the sheep uterus. These studies concluded that the sheep uterus was robust and could tolerate at least one hour of warm ischemia.[53] The first allogeneic sheep UTx was performed on 12 sheep where cyclosporine was used for immunosuppression. Five of the animals had embryo transfer and three pregnancies were reported leading to one livebirth from a cesarean section.[54] However, another group reported some organ rejection and necrosis problems after an orthotopic allogeneic UTx in the sheep, even after immunosuppression.[55]

Following the developed surgery techniques on the sheep and the pig animal models, further UTx investigation was conducted in non-human primates, including on macaques and baboons due to their near resemblance to the human uterus in anatomy and physiology. The first UTx on a baboon model was performed in Saudi Arabia with an autologous orthotopic UTx that demonstrated positive results in graft survival after 6-12 weeks.[56] Prof. Brännströms group also reported successful autologous UTx in baboons and assessed the long-term results. In brief, five of the nine animals survived the UTx, of which two animals resumed menstruation.[57] The same group reported an allogenic UTx in baboons where all the recipient animals showed signs of mild rejection. There was no kidney damage despite the animals having high tacrolimus levels in the blood. Nevertheless, the authors

suggested the need to optimize the immunosuppression regimen.[58] A Japanese team then reported UTx on two macaques where one animal died due to renal failure, while the other animal was healthy and resumed menstruation post-surgery.[59] The same group was successful with an imaging technique to evaluate blood flow following transplantation using indocyanine green. They suggested that a unilateral anastomosis of one uterine artery and one uterine vein provided sufficient blood to the uterus grafts following UTx.[60]

Human studies

The first clinical uterus transplant was performed in the year 2000 in Saudi Arabia with little prior UTx research or any significant preparation except for the above mentioned short-term baboon UTx. A group of surgeons decided to try the UTx procedure as a fertility treatment for a 26 year old hysterectomized woman who received the uterus of a 46 year old woman undergoing hysterectomy due to benign ovarian cysts. After blood and tissue typing, the UTx was performed with no major complications initially. However after three months, the graft showed necrosis with thrombosed vessels that lead to the removal of the graft.[56] The second human UTx case was then conducted by a group in Turkey. Similarly to the Saudi Arabian case, no previous UTx research or training had been conducted by the group prior to the human trial. The patient was a woman diagnosed with uterus aplasia and the donor was a young, nulliparous multiorgan donor. The patient had menarche and regular menstrual cycles after the transplantation surgery.[61] Multiple embryo transfers were attempted, and clinical pregnancy achieved which suggested that embryo implantation can take place in a transplanted uterus. However, no live birth from this patient has been reported.[62]

The first successful UTx with a livebirth was performed at Sahlgrenska University Hospital by a surgical team lead by Prof. Brännström after more than a decade of translational research.[63] This has since been followed up with multiple subsequent livebirths from other patients operated by the same team with improved operating protocols.[64-66] To date, there are 11 babies born in the Swedish clinical trials and there

are now multiple additional livebirths reported from around the world.[67-72] UTx has thus been proven to be a novel fertility treatment for the earlier believed “untreatable” condition of AUFI. However, the procedure is still considered an experimental treatment, and protocols are continuously being optimized and improved, e.g. regarding the donor source (live or deceased donor),[63,69,70,73] the use of robotic surgery to reduce operating time and blood loss,[66,74-76] positioning of the vascular anastomosis[77,78] and organ preservation methods.[79-81]

Risks with uterus transplantation

In general, there is a shortage of organ donors all around the world.[82] This also implies for UTx since the strict donor criteria for UTx limit the availability of donor uteri, even when using deceased donors. Furthermore, it is common that vital organs such as the liver, lung, kidney or the heart are the first organs to be explanted to limit organ damage associated with the warm ischemia time. The uterus would not be considered a high priority organ as it is not essential for survival. Therefore, a deceased donor uterus may have experienced a longer warm ischemia than other organs that may adversely affect the outcome after transplantation. Thus far, most of the current successful UTx cases that resulted in livebirth came from a living donor. These operations are easier to plan logistically, and the donor uterus can be thoroughly examined prior to the donor surgery. However, the donor surgery is a risky procedure since it involves operating deep into the pelvis as the uterus needs to be isolated with long intact blood vessels, which are in anatomical proximity to the uterus, in order to facilitate a vascular anastomosis upon engraftment and with undistributed ureteric function in the donor.[83] The recipient further risks significant adverse side-effects from the carefully curated immunosuppression regimen to prevent organ rejection, including infections, renal failure or embryo implantation failure.[84-86] The last point is important to consider since the whole reason for UTx is to restore fertility. Excluding donor or recipient death, or risks related to unintentional surgery inflicted injuries to other organs such as the ureter or the colon, the worst case scenario is the

possibility of organ rejection. Although immunosuppression has advanced to a great extent, the risk of organ rejection is still significant. A part of the aforementioned risks could potentially be overcome with a tissue engineered organ that was constructed using the recipient's own cells.

Tissue engineering

An abridged definition of TE is the construction of a viable organ in a lab that can be used for transplantation to restore and/or improve the function of a damaged organ in the recipient. This may be done with a 3D scaffold along with cells and growth factors (GFs) that facilitate in-vivo remodulation and function post transplantation.[87] In the clinic, TE constructs have been used in vascular grafts,[88] bone[89] and skin regeneration applications.[90] Additional TE solutions have been used for urethra,[91] breast[92] and trachea.[93] However, to achieve proper TE for more complex organs is challenging. Therefore, much preclinical research is focused on the construction of TE organs suitable to replace an organ donor in a transplantation setting. If successful, such a feat would revolutionize the organ source for transplantation as an organ or a tissue could be constructed from an immune-inert scaffold and the patient's own cells, thereby eliminating immunological barriers such as human leukocyte antigen compatibility and immunological donor-recipient matching. This may be used for essential and elective surgeries both of which would improve quality of life of the patient.

As summarized briefly below, there are a number of different scaffolds that may be appropriate to use for TE applications, and they all have their respective pros and cons. However, all scaffold types should optimally be immunologically inert, not contain any donor nuclear material and facilitate regeneration.

Polymer scaffolds

Polymer is derived from the Greek words “poly” and “mer” meaning many and parts, respectively. Scaffolds made of polymers can be either natural or synthetic. Natural polymeric scaffolds are derived from a natural source where the components can be found in the body and could thus be absorbed after implantation. Examples of natural polymer scaffolds include those made of collagen, hyaluronate, gelatin, chitosan, fibrin and silk, among many others.[94] The major advantages of these scaffolds are that they are biocompatible, biodegradable and have shown to be able to facilitate cell adhesion and migration, while the disadvantages are batch variation and limited mechanical properties.[95] Synthetic polymeric scaffolds are made of either fully synthetic monomers (e.g. poly-caprolactone and poly-dioxanone), or semi-synthetic sources (such as poly-lactic acid, poly-glycolic acid or poly-glactin, among others).[94] The advantages with these types of biomaterials are reproducible batches, good mechanical scaffold properties and sterility. However, the disadvantages with these types of biomaterials are that they can cause an immune response when the polymers are broken down into monomers.[96] There is a recently published report of a polymer based uterus scaffolds that resulted in livebirths in rabbits,[97] further described in detail later in this thesis.

Porous scaffolds

Porous scaffolds may be derived from a natural source (i.e. from marine resources such as Porifera sponges)[98,99] or synthetic sources such as hydroxyapatite composites, gore-tex or dacron among others.[100] The advantage using these scaffolds is the absence of an inflammatory response post in-vivo engraftment.

Hydrogel scaffolds

Hydrogels are gels made of large cross-linked molecules that can absorb a lot of water. On account of their hydrophilic nature, they are

somewhat similar to native organs. Hydrogels can be produced either from digested organ matrices or from semi-synthetic polymers like polyethylene glycol that has biodegradable functional groups. One interesting application for hydrogels is its use for 3D bio-printing of organs using a biocompatible matrix along with cells. This is yet to be further optimized for smaller constructs, but has been used for tissues like bone and cartilage.[101,102]

Extracellular matrix derived scaffolds

Extracellular matrix (ECM) derived scaffolds are generated from tissues or organs after the successful removal of cells in a process named decellularization (DC). To the best of my knowledge, the first ever tissue engineered ECM scaffold was performed in 1977 where a decalcified bone was used as graft after it had been seeded with stem cells. The construct was shown to stimulate the repair of a defective knee joint in a rabbit model.[103] Since then, TE ideas, protocols and technologies have improved significantly. Currently, TE studies using decellularized tissue as scaffolds have been performed in a multitude of organs and in a great variety of animal models, including studies on the heart,[104] lung,[105] urethra,[106] liver[107,108] and more recently the uterus.[109-119] The advantage of using the ECM as a natural scaffold is that it is composed of the same building blocks of the organ of interest, and it can give structural support and provide essential GFs for the migration and proliferation of cells during remodeling.

Components of the extracellular matrix

The ECM is comprised of a myriad of components, all of which are very important for the natively present cells. The most prevalent and well-studied components of the ECM are collagen, elastin, sulfated glycosaminoglycans (GAGs), fibronectin and laminin which are summarized below.

Collagen is a protein that makes up around 85% of the ECM. There are around 28 types of collagen, all of which give structure, strength

and shape to the tissue. Collagen type I is the most abundant collagen in the human body and is principally located in bones, tendons and skin but is also a major component in other tissues.[120] The uterus predominantly consists of collagen types I, III, IV and V.[121] More specifically, collagen types I and V are predominantly distributed around cells while type III are organized around cell bundles.[122]

Elastin is an important protein that is responsible for elasticity, contractibility and load bearing properties and co-exists with collagen in all organs. Naturally, it is important in the uterus for its ability to expand during pregnancy. However, it is also responsible for signaling, giving mechanical stability and organization of the ECM structure.[123] The distribution of elastin shows variations in different locations of the uterus where only small amounts of elastin can be found in the inner smooth muscle layer close to the endometrium. However, there is a gradient increase of elastin towards the outer myometrium with a significant distribution of elastin at the serosal boundary. There is also an increasing gradient of elastin in the lower part of the uterus especially in the uterine cervix.[124]

The GAGs are complex carbohydrates with the addition of an amino group that are negatively charged. The viscosity and hydrophilicity of GAGs make them retain water and will then provide an effective storage place for GFs, chemokines and cytokines. They are present on both the ECM structures and the cell surfaces.[125] The uterus is rich in GAGs where the endometrium has a predominance of chondroitin sulfate along with smaller amounts of dermatan sulfate and heparan sulfate, while the muscular myometrium has a high level of hyaluronic acid.[126]

Fibronectin comes second, after collagen, in the abundance of specific ECM components in the body. A soluble form of fibronectin is found in the circulatory system and an insoluble form is found in the ECM. The dimeric form of the protein can bind to other ECM components such as collagen, heparan, fibrin among others.[127] In the uterus, it is well distributed everywhere, but with the majority found mainly around cell bundles. Fibronectin also plays an important role in pregnancy where it

binds to cleaved collagen during pregnancy and is a key factor in collagen removal during the remodeling after pregnancy.[122]

Laminin is a basement membrane glycoprotein that has 11 recognizable molecular chains, found as alpha, beta and gamma subunits. Laminin has an affinity towards collagen type IV and heparan sulfate. It is essential for cell adhesion, migration and proliferation while also playing an important role in cell signaling.[128] The uterus has, in relation to other organs, a high amount of laminin, especially in the endometrial layer where it plays an important function during embryo implantation.[129]

Decellularization

The DC is the process of removing cells and cellular material (including nuclear material) from organs and tissues to create ECM derived scaffolds. There are a number of different methods to be used for this procedure. Since any DC process not only remove cellular components, but also results in the damage of the ECM structure and its mechanical properties, it is important to find a fine balance between effective removal of donor cellular material while retaining the important ECM components. The choice of the DC protocol employed depends completely on the type of tissue, its cellularity, thickness and mechanical properties.[130] There are broadly two important factors that impact the DC process: physical and chemical factors.

Physical factors

Temperature is useful since freeze-thaw cycles disrupt the cell membranes in the tissue. This is often followed up with additional other methods for DC, and thereby enhancing the removal of cellular components. Temperature is also something to be considered while using detergents or enzymes as the efficacy of these strategies is

temperature dependent. Like every process, the free-thaw cycles can also irreversibly damage the ultrastructure of the DC tissue.[131,132]

Agitation is the method where a DC reagent is in constant contact with the tissue during brisk stirring. The DC reagents penetrates the tissue through passive diffusion, thereby disrupting the cell membranes and dislodging them from the ECM. This method is often deployed for non-vascular tissues like cartilage or bone, or pieces of soft tissue.[133]

Perfusion is a method when a DC reagent is forced through the intact vasculature of the whole organ. It is an effective method for soft organs with vascular pedicles that can be cannulated, since the blood vessels reach the inner most parts of the tissue where, for example, DC protocols using passive diffusion cannot be reached. The advantage with this method is the effective removal of cells using lesser concentration of detergents or a reduced exposure time to DC reagents. This consequently reduces the ECM damage. Furthermore, the created ECM scaffold after a perfusion-based DC protocol also have an intact whole organ structure with remaining conduits of the vasculature. These conduits can be beneficial for cellular reconstruction experiments, and used in future vascular anastomoses protocols for transplantation studies in-vivo.[104,134]

Ultra sonication is the use of ultrasound that causes physical vibration of the tissues. It is especially useful for DC of robust or dense tissues where the cells are dislodged from the ECM through the use of just mechanical force. The tissues are usually immersed in cold buffers in order to reduce heat from the sonication process that could inadvertently damage the ECM.[135]

Chemical factors

pH dependent solutions such as acids and alkalis act as DC reagents by lysing the cells due to the solubilization of the lipid layer and parts of the cytoplasm. The most commonly used acids include peracetic acid which is also widely used as a sterilization agent of ECM derived

scaffolds. A commonly used alkali includes sodium hydroxide. Although these reagents are efficient, this method for DC is known to significantly damage the structural ECM components, in particular collagen, GAGs and GFs.[136]

Tonicity is the osmotic pressure gradient between two liquids separated through a semipermeable membrane which in the case of DC represents the cell wall. A hypertonic solution (e.g. salt water) has higher salt concentration than the cell, thereby making the cell shrink. Alternatively, a hypotonic solution (e.g. deionized water) has lesser salt concentration than the cell, and thereby water is forced into the cell through osmosis and causing it to swell and ultimately burst. Hence, tonicity is an effective DC strategy.[135,137]

Solvents include the primary substance in which the actual DC reagent is dissolved in. The most widely used is deionized water. However, there are organic solvents e.g. alcohols or tri-n-butyl phosphate that effectively dissolve lipid-lipid or protein-protein bonds respectively. High polar solvents such as dimethyl sulfoxide (DMSO) may be effective DC reagents when interchanged with a hypotonic solutions since the osmotic shocks will disrupt cellular membranes.[138]

Detergents are surfactants that have both hydrophilic and hydrophobic groups that influence the tissues during DC, depending on what detergent type and its concentration. Ionic detergents such as sodium dodecyl sulfate (SDS) and sodium deoxycholate (SDC) are effective and popular DC detergents since they break protein-protein bonds and can thereby effectively solubilize the cell-ECM bonds and cell-cell bonds.[139-141] Non-ionic detergents (e.g. Triton X-100) are milder than ionic detergents in regards to the damage inflicted on the ECM during the DC process but may require longer exposure times to establish an effective DC of the tissue. Non-ionic detergents act by breaking protein-lipid and lipid-lipid bonds.[142] Zwitterionic detergents (e.g. CHAPS) have the properties of both ionic and non-ionic detergents and when used for DC can maintain the structural ECM integrity well. However, it tends to leave an abundance of cytoplasmic remnants in the tissue, and these remnants can be detrimental to the scaffold application.[134,143]

Enzymes are biological catalysts that facilitate chemical reactions. The enzymes used for DC are predominantly serine proteases and nucleases. Trypsin is the most used protease for removal of cells and acts by cleaving the bonds on arginine and lysine amino acids. This facilitates the removal of cell-ECM bonds while also disrupting some of the ECM protein structures.[144] Nucleases such as deoxyribonuclease (DNase) cleave oligonucleotides thereby breaking down deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) strands to smaller fragments, which then could be washed away with another DC reagent or solvent.[144,145]

Chelating agents (e.g. ethylenediaminetetraacetic acid) bind to metal ions such as calcium and magnesium and can thereby disrupt cell-ECM bonds. It can also be used for protease-rich tissues (i.e. pancreas) to inhibit the enzymes thereby reducing tissue damage.[146,147]

Evaluation of decellularization

Ideally, successful DC is achieved when all the cells, with their membranes and organelles as well as the nuclear material have been removed, while as much as the ECM is preserved, including proteins, GFs and cytokines. The preliminary step to evaluate successful DC is to use histological analyses to evaluate the sectioned DC tissue and look for nuclear and cellular residues, or lack thereof. Electron microscopy could be used to study the ultrastructure of the ECM before and after the DC process. These preliminary assessments of DC could then be followed up by more detailed analysis to evaluate the remaining ECM structure, its specific content of various components and/or other important scaffold attributes (several examples are summarized below).

The **DNA** content in the ECM derived scaffold is very important since small and long strands of donor DNA remnants could cause an immune response after implantation. Smaller DNA strands can recruit (immune cells) as it signals apoptosis, while larger DNA strands could result in a foreign body reaction.[148,149] Although the recommended threshold of acceptable remaining donor DNA in DC tissue is “less than 50ng of DNA

per mg of dry DC tissue",[130] that statement was made with no references and could merely be an arbitrary statement. This may very likely be tissue specific and needs further investigation. Another DNA criterion for DC tissue is that the remaining DNA fragments should be less than 200 base-pairs long, since such fragments may be degraded by the immune response post engraftment.[150]

ECM proteins and GFs are important components in ECM derived scaffolds. The DC process should always strive to preserve these as much as possible. Hence, the remaining quantity of these constituents are often assessed in the DC tissue. The amount of collagen, elastin, GAGs, fibronectin, laminin and other ECM components are very important in order to achieve proper recellularization (RC) of the scaffolds. The amount of GFs that remain in the DC tissue also play a vital role as they promote seeded cells in downstream RC and transplantation applications.[151] The most straightforward method to quantify GFs is by immunoassays. Although the most extensive method to determine every ECM component, including GFs, is by proteomics, it should be noted that GFs are denatured before the analysis and does not show if the detected GFs are functional.[111,152,153] Nevertheless, there are assays that could demonstrate functionality of specific GFs responsible in regards to physiological activities such as neurogenesis[154,155] and angiogenesis.[156]

Mechanical properties of the scaffold are crucial physical attributes where the DC scaffolds need to have similar properties as the native tissue it should replace or repair. This is especially important in tissues submitted to mechanical stress such as blood vessels, esophagus and the uterus where the organ's mechanical strength have to be retained in order to have functionality after engraftment. The mechanical strength can be measured using sheer-stress moduli, young's modulus, expansion ratio, elastic modulus etc.[111,157,158]

Sterility of the scaffold is, naturally, important if it ever will need to be recellularized and/or transplanted. Scaffolds produced by a DC process are usually sterilized by e.g. using the acidic-oxidizing per-acetic acid[159]

or by gamma radiation.[160] However, both these methods can inflict significant damage to the ECM.[161]

Recellularization

The RC is the process of seeding cells to a DC scaffold with the goal to regenerate a functional construct that could repair or replace a damaged tissue in-vivo. The requisites for a successful RC are a well evaluated DC scaffold,[130] a good cell source,[162,163] a suitable cell culture media with GFs[164] and a good cell culture environment in-vitro such as an incubator for smaller tissues or a perfusion bioreactor for whole organs.[105,165]

Cell source

The cell source used for RC is an important consideration for the success of creating a functional bioengineered tissue. Optimally, the cell source should be from the patient to be transplanted to avoid a potential immune rejection event after transplantation. The cells should also be easy to obtain and be available in considerable quantities. It is further advantageous if the cells have a high proliferative potential, are easy to expand in-vitro and are safe to use for a clinical application. However, there are two general principles to consider that depends on the underlying purpose of the RC: a) the added cells are used with the aim to recreate and/or differentiate them into the specific tissue, or b) they are used to stimulate homing of endogenous stem cells and stimulate in-situ tissue regeneration by secreting paracrine factors after transplantation. Additionally, the choice of cells may also induce distinct immune response patterns.[166] Broadly, there are two sources of cells, namely somatic cells and stem cells.

Somatic cells

Somatic cells specifically refer to the differentiated cells isolated from tissues of humans or animals. These cells have undergone a low

number of population doublings and therefore represent the source tissue more accurately as compared with a cell line that after many passages is adapted to an in-vitro milieu. The advantage of using somatic cells is the retention of functionality and the specific phenotype of the tissues or organs they are isolated from, making them the ideal cell type to use for reconstructing a specific tissue type. They are also better than using cell lines as immortalized cells may not be clinically approved because of a potential cancer risk, since the cells are manipulated to not lose their ability to proliferate (senescence). Somatic cells are easier to obtain compared with stem cells, and may be isolated from skin or from the endometrium. However, they are hard to isolate from organs such as the liver or the kidney without general anesthesia. Somatic cells also tend to stop growing after 40 to 60 population doublings. Unlike fibroblasts that grow very fast in-vitro, specific cell types such as epithelial cells or hepatocytes do not grow as fast and are also prone to rapid phenotypic changes.[167] All these disadvantages suggest other cell sources should be investigated.

Embryonic cells

A zygote is a totipotent cell that can give rise to a whole organism while a pluripotent cell is a cell from the inner cell mass of a blastocyst that could differentiate into the three germ layers: ectoderm, endoderm and mesoderm. An embryonic pluripotent cell is more commonly known as an embryonic stem cell. These cells are generally derived from donated unused embryos created in abundance during an IVF treatment. The advantages with using embryonic stem cells for RC are the pluripotency which means that they could differentiate into almost any type of tissue. They are easy to obtain from IVF clinics with the right ethical approval and could rapidly be expanded to vast numbers in-vitro. The downside is risk of teratoma formation after transplantation,[168] and the questions have been raised with respect to working with embryos for ethical and/or religious reasons.[169,170]

Induced pluripotent stem cells

Pluripotent cells can not only be obtained from zygotes but also from reprogrammed (induced) somatic cells by the introduction of the

Yamanaka transcription factors.[171] Induced pluripotent stem cells have a main advantage of not causing an immune reaction when transplanted as they can be harvested and reprogrammed from cells taken from the recipient (autologous cells). They also have a high proliferative capacity that can be cultured to billions of cells from a relatively small starting cell number. This makes them an attractive cell source for RC in various TE studies. Additionally, they have the potential to differentiate into any cell type. As with their embryonic counter source, they can potentially cause teratomas after transplantation. However, with recent advancements, developed differentiation protocols can reprogram these pluripotent cells into a specific lineage, thus making them safer to use.[172] Interestingly, a small part of the human population considered to be “super donors” could donate to a large part of the population, is elaborated later in this chapter.[173] The induced pluripotent stem cells have been used to RC rat lung tissue where human cells attached and proliferated similar to a human lung environment.[174] They were also used to RC liver scaffolds where a hepatic phenotype was stimulated using a 3D microenvironment of ECM derived scaffolds.[175]

Multipotent stem cells

Multipotent stem cells are progenitor cells that are more mature than pluripotent cells as described above. They have the ability to differentiate into a limited number of lineages. The two most commonly used multipotent stem cells are mesenchymal stromal/stem cells (MSCs) or adipose derived stromal/stem cells. Both these cell types are relatively easy to obtain and has the potential to differentiate into chondrogenic, osteogenic, adipogenic and myogenic lineages. The advantages of using these cells are the relatively easy isolation procedure, their basic culture requirements and the ability to be expanded in-vitro. These cell types have also been approved for clinical use and they have advantageous immunomodulatory effects and autologous characteristics when grafted. The downsides of using these cells are the inability to differentiate to any type of cell, and complications related to the isolation procedure such as harvest site infection. Furthermore, elderly patients have lower MSC population

reservoirs that may become inadequate for the cell numbers required for RC and organ reconstruction for this patient group.[176-178]

Methods of recellularization

The RC has been conducted using a multitude of methods in different milieu, from simple techniques that include injecting a very large number of cells into the organ following static cell culture conditions, to more sophisticated methods of administering cells through different organ/tissue specific conduits with the use of perfusion bioreactors. Each method used for RC can be successful, but much of it depends on the size and thickness of the tissue to be recellularized.

Static recellularization methods

Static RC is the standard RC method deployed by most studies that show a proof of concept of RC.[179] The word “static” refers to that there is no agitation or perfusion during cell culture period directly after the cell seeding procedure. Rather, the RC scaffold is kept undisturbed in the incubator, usually submerged in cell culture media. This is usually also where the actual RC takes place by the administration of specific cells, commonly via direct injections using a syringe. Static conditions may not always result in a homogenous cell distribution, especially if the organ is thick or consists of a dense scaffolding structure. This may lead to apoptosis in the middle of the construct due to limited nutrient diffusion and an accumulation of toxic metabolites.[180] Depending on cell types used for RC, the static RC methods could be advantageous for certain stem cells that grow well under hypoxic conditions.[109] Other improvements for static RC conditions may include the use of transwells (TW) to increase media access to the construct[147] or the use of hydrogels to seal the cell injection site on the scaffold after the administration to decrease cell leakage.[181]

Dynamic recellularization methods

Dynamic RC methods involve some kind of physical force such as agitation, stirring or cell perfusion to improve nutritional and metabolite

exchange in the RC scaffold to enhance cell adhesion, migration and proliferation. A bioreactor is a perfusion machine that traditionally is used to keep an organ viable in cold temperature between donor and recipient in an organ transplantation setting.[182] As the knowledge in the field expanded, bioreactors are now being considered for RC of whole or partial organs.[180] Bioreactors are often custom made for specific organs. For RC of simpler organs such as blood vessel and cartilage, spinner flask bioreactors can be used successfully where, for example, the perfusion medium flows on the outside of the organ, while cells in the luminal space are fed with a different media under constant spinning from a stirrer.[183] Highly vascularized organs like the liver, lungs, kidney and uterus require more complex bioreactors with inlets and outlets for the arteries and veins, respectively, with constant monitoring of the perfusion loop, the vascular resistance and temperature. Additionally, attention to a change in the perfusate concentration of oxygen, carbon dioxide, glucose, pH and lactate is also critical to maintain an optimal RC environment and reduce the buildup of detrimental metabolites during the process.[81,165,184] These factors may to some degree be regulated by the basic components of cell culture media used for perfusion solution. Generally, culture medium consists of carbohydrates, amino acids, lipids, vitamins, trace metals and buffers, all of which are the underlying vital components.[185] It is most of the time also supplemented with antibiotics to prevent contamination[186] and serum, or with a serum replacement, to provide albumin, GFs and hormones.[187] Osmolality is also a very important aspect to the perfusate since it affects cell homeostasis and edema. This may be specific to certain cell types, since they differentially depend on the amount of ions absorbed or released, specific to the organ of interest.[188] Additionally, oxygenation of the medium is important so that enough oxygen can reach the deep layers of the RC scaffold. Synthetic oxygen carriers such as perfluorocarbons have been used in cell culture to improve the oxygen concentration dissolved in culture medium.[189] These oxygen carriers was also used in the clinic as a blood substitute for 186 patients suggesting that they can be used safely in-vivo.[190] Oxygen carriers were also studied to reduce ischemic damage, suggesting that they

may have a protective effect during cerebral ischemia by preventing the vasoconstriction.[191]

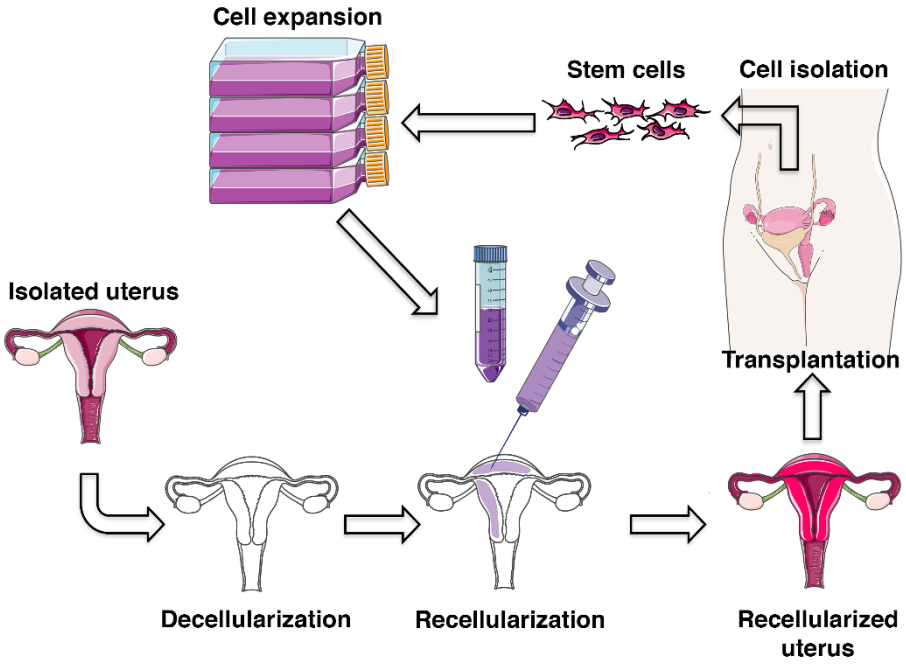

Figure 4. Overview of decellularization and recellularization

Evaluation of recellularization

A straightforward and frequent method to visualize seeded cells after RC is through histological analysis and microscopy. However, if the DC is incomplete and new cells are added to the ECM scaffold, it is difficult to distinguish between residual donor cells and the seeded cells that were used for RC. It is therefore likely that many studies overestimated their RC efficiency. A solution to this can be to tag the cells used for RC with a fluorescent molecule (e.g. green fluorescent protein) that distinguish them from any potential donor cells left in the scaffold.[112] Qualitative analysis could help to identify if the cells are distributed homogenously, and quantitative analysis should be performed to evaluate the cell density in the scaffold in an unbiased manner. Electron microscopic analysis is a powerful tool to elucidate cell-ECM

interactions at the ultrastructural level.[181] Immunoassays are effective methods to determine cell phenotype and could be coupled with other methods such as gene expression analysis to verify the finding.[192] Mechanical tests could be deployed to see if the physical properties may have improved after RC and to investigate if the construct is comparable with the native tissue. Another method, which is a very comprehensive strategy to evaluate RC, is to conduct proteomic and genomic analyses on the construct to determine very detailed characteristics of the scaffolds and the cells.[193]

Immune response towards tissue engineered grafts

Tissue engineered grafts are composite scaffolds that are generated by unique DC processes, different sterilization protocols and distinct RC strategies, all of which can trigger or modulate the immune response after engraftment.[194] The uniqueness of each graft type makes it complicated to draw parallel conclusions between studies. However, after the implantation of a tissue engineered graft, a constructive remodeling generally occurs that is orchestrated by infiltrated host cells. This event takes place simultaneously to the biomaterial degradation (if a degradable scaffold is used). Both these events are correlated with successful long term outcomes.[195]

Immunogenicity of decellularized grafts

The DC process changes the ultrastructure of the tissue due to the effect of the various DC reagents used for the scaffold production. This exposes damage-associated molecular patterns (DAMPs) which are fragmented ECM molecules dislodged from e.g. heparan sulfate, hyaluronic acid and fibronectin, among others. Cellular components could also act as DAMPs, including nucleic acids, high mobility box group 1, heat shock proteins and S100 molecules. All these molecules

are known to be able to induce an immune response after engraftment via the activation of toll-like receptors and receptors for advanced glycation end products proteins.[196-198]

Extracellular matrix based DAMPs

It has been shown that chemotaxis of neutrophils and monocytes can be triggered by fragments of ECM molecules acting as DAMPs following engraftment. This includes collagen[199], elastin[200] and fibronectin.[201] Cytokine and chemokine production along with dendritic cell maturation could be triggered by damaged hyaluronic acid molecules[202,203] and fragmented heparan sulfate structures within the ECM.[204] It was also documented that laminin-based DAMPs induced a matrix metalloproteinase (MMP) 9 release in leukocytes that could facilitate the chemotaxis for inflammatory cells. Although the physiological role of MMP-9 secretion from immune cells is not known, it is hypothesized that the production of MMP-9 aids in their infiltration.[205]

Cell based DAMPs

Cellular-based DAMPs are released from dead cells and could, for instance, be a result of the DC process. For example, high mobility group box 1 proteins are chromatin proteins released from dying cells that can induce cytokine expression in immune cells and macrophage activation.[206] Heat shock proteins may result in a cytokine induction and a stimulation towards dendritic cell maturation.[207,208] S100 proteins released into the ECM from cell lysis can also lead to increased cytokine expression and chemotaxis of immune cells.[206] Furthermore, DNA fragments are well known DAMPs and are associated with dendritic cell maturation and macrophage activation.[148] For these reasons, it is generally regarded that low quantities of DNA that could act as DAMPs in DC tissue is advantageous. A somewhat arbitrary amount of 50ng double stranded DNA per mg dry scaffold was stated in a highly cited review article has become the general norm for scaffolds created by DC. Surprisingly, this amount was mentioned without any data or reference to back it up.[130] However, there is evidence suggesting that the fragment size is important from an immunological perspective.

Thus, it has been suggested that the size of residual DNA fragments should be less than 200 base pairs (bp) in size to avoid a detrimental immune reaction. This maximum size limit is correlated to when apoptotic cells break down their DNA in a controlled fashion to fragments around 180 bp, without causing a negative immune response.[209] Albeit, even small DNA fragments as low as 24 bp could activate macrophages.[210] One often overlooked cellular component is RNA remnants, likely because of its rapid degradation. However, it should nonetheless be considered as a potential DAMP.

Immunogenicity of recellularized grafts

The overall immunogenicity of RC constructs is largely unknown. Besides the broad variability of the various DC scaffolds, RC constructs have further added variables as a consequence of the RC method employed, and the type of cells used. Very few studies investigated the immune response affected by the different types of RC methods. Nonetheless, there are some reports that assessed the immune response towards various type of cells commonly used in TE as summarized by Ochando et al.[166]

Immunogenicity of embryonic stem cells

The undifferentiated pluripotent nature of human embryonic stem cells may be regarded as non-immunogenic as a result of not inducing a T-cell response, despite being allogeneic.[211] Yet, in mouse models, allogeneic embryonic stem cells were rejected, an event that evolved faster following repeated administrations, possibly due to immunological memory mechanisms.[212] Major histocompatibility factor (MHC) class 1 expression is relatively low in embryonic stem cells, yet somehow, it does not seem to trigger natural killer (NK) cells.[213] However, due to the potential allogeneic nature, these cells would require the administration of immunosuppression after engraftment. Therefore, it may be an advantage to find an alternative cell source for RC and TE applications.

Immunogenicity of induced pluripotent stem cells

To overcome disadvantages incurred by the allogenic nature of embryonic cells, induced pluripotent stem cells could be used as they may be isolated from the prospective recipient, and thus, would be of autologous origin. Still, the process of creating induced pluripotent stem cells from cellular reprogramming may lead to epigenetic changes that risk unpredictable phenotypic variations. The MHC class 1 expression levels are generally low as a result from the reprogramming procedure.[214] However, one downside with using these cells for TE is the cost and logistics required for the isolation and expansion process which would have to be repeated for every single patient in need of a personalized TE organ/tissue. More recent advancements have discovered that some donors are human leukocyte antigen homozygous, more popularly called “super donors”. As mentioned in an earlier in this chapter, cells isolated from such donors could be reprogrammed into induced pluripotent stem cells and used as a source for a significant number of potential recipients. It is estimated that 140 of such “super donors” could cover about 90% of the world’s population.[173]

Immunogenicity of adult multipotent stem cells

Adult/tissue-specific stem cells have been used in TE on a multitude of organs over the last three decades.[215] The scientists working with adult cells have been studying the properties of the cells in terms of differentiation ability. Yet, the immunogenicity is still largely unknown. It is known that hepatic stem cells can help with the reconstruction of livers, possibly because of the spontaneous regenerative ability of the liver and its constituent cells.[216] They are also shown to reduce an inflammatory response by suppressing dendritic cell maturation, NK cell activity and T-cell proliferation by releasing prostaglandin E2.[217] However, the complete proliferative ability of adult stem cells from other organs may not be as good as the liver. In case of patients with a diseased or absent organ that is in need of a therapeutic tissue engineered construct, it would be difficult (or impossible) to isolate tissue-specific stem cells for this application.

Multipotent MSCs are considered to have a great advantage over many other cell sources for their accessibility and rapid in-vitro cell expansion characteristics. The increased use of MSCs in the clinic have shown that autologous and allogenic sources are safe to use and that they act immunomodulatory after transplantation.[218] They have also shown to induce an immune-tolerance for tissue engineered constructs.[219] Nevertheless, the molecular patterns of the MSCs depend on the source tissue from which the cells are isolated from.[220] With regards to uterus TE applications, MSCs are a promising source of cells for RC since a patient in need of a uterus transplant is likely to lack a native uterus to harvest cells from.

Uterus tissue engineering

Initial uterus TE studies mainly concentrated on the endometrium to investigate the effects of hormones and/or to study interactions between different uterus cell types in co-cultures. Most of the studies were performed using collagen matrix, sometimes with the addition of Matrigel. More specifically, these studies were performed in-vitro using cells from the rat,[221] the rabbit[222] and human.[223-227]

Uterus tissue engineering in rodent models

Recently, uterus TE has been studied more intensely and included the use of DC scaffolds to reconstruct uterine defects. Below is a brief summary of the most relevant published articles for this thesis, and a more extensive summary of uterus TE is detailed by Hellström et al.[228] , and by Campo et al.[229]

Santoso et al. published a study in 2014 where DC uterine tissue segments from rats were analyzed.[118] They compared three different DC protocols with 1) SDS, or 2) Triton X-100, or 3) high hydrostatic pressure. They concluded that protocols 1 and 3 were better than protocol 2. Therefore, when they conducted the in-vivo experiments

they only used these two uterus scaffold types. The full thickness 15mm × 5mm DC scaffolds were repopulated with host uterine cells after engraftment. Pregnancy tests performed 30 days after engraftment revealed that the scaffolds were able to provide structural support during fetal development.

The TE of whole rat uterus was first reported in 2014 by our group[111] and a group in Japan.[116] Hellström et al. optimized three DC protocols for whole rat uterus, where the first two protocols were based on Triton X-100 and DMSO, dissolved either in deionized water or in phosphate buffered saline (PBS). The third protocol used the ionic detergent SDC. All the scaffolds were characterized for remaining ECM proteins and the scaffolds mechanical properties.[111] This DC study was then followed up with a further investigation of RC and transplantation. Uterine patches (10mm × 5mm) were RC with fluorescently labeled MSCs together with primary uterus cells and were then transplanted to replace a full-thickness uterine wall injury.[112] The fertility rates were restored in grafts from two of the three protocols when tested 6 weeks after engraftment. Hence, the Triton X-100 and DMSO protocols were better than the SDC protocol. Miyazaki and Maruyama tested an SDS based protocol that had previously been optimized for the liver.[116] The DC whole uteri were recellularized with neonatal- and adult uterine cells and MSCs and then placed them in a perfusion bioreactor. Following that, uterine patches were cut out from the RC uterus scaffold and were transplanted in a similar way as described in the Hellström et al., study. This report also assessed functionality trough pregnancy tests conducted 28 days after engraftment. The number of fetuses in the grafted uterine horns were significantly lesser than in normal pregnant animals. However, the fetuses developed normally in the transplanted horns. This study was followed up by another transplantation study using DC uterine patches generated from the same SDS protocol but without any cells.[115] This study revealed that the orientation of the ECM during transplantation is important to achieve the correct morphology during uterus regeneration and potentially result in uterine structural disease. However, when pregnancy tests were conducted on these

animals, properly oriented and disoriented grafts had the same number of fetuses growing in the operated horns.

A similar process of TE was performed in a murine study, where DC uterus segments were grafted to the uterus of either ovariectomized mice or STAT3 conditional knockout mice. The results indicated that the regeneration process was not dependent on the ovarian hormones but showed STAT3 playing an important role.[113] Nonetheless, these studies need to be further investigated to understand the mechanisms and also need to be evaluated again before scaling up to larger animal models.

Uterus tissue engineering in lagomorph models

Uterine TE was also reported in a rabbit model.[230] In this study, non-synchronous (i.e. a non-proliferative endometrium) and synchronous uteri (after ovarian stimulation) were DC using a combination protocol based on SDS, Triton X-100 and DNase. The assessment of DC showed the successful cell removal and a reduction in the total protein content. The DC endometrium was then isolated using microdissection and was further solubilized into a hydrogel. This ECM endometrium-specific hydrogel was thoroughly characterized using proteomic analysis with respect of the two different hydrogels form synchronous and non-synchronous endometrium. Results showed that there were different quantities of ECM proteins such as collagen type III, collagen type IV and laminin among many others. The study concluded that synchronous endometrial hydrogel supported rabbit embryo development in-vitro better than hydrogels form non-synchronous endometrium, suggesting that donor hormonal stimulation prior to uterus DC procedure may be beneficial.

The more recent study on uterus TE using a rabbit model describes the use of a biodegradable PGA/PLGA polymer based scaffold.[97] Although, this study does not use DC tissue as scaffold type, and thus is outside the main scope of this thesis, it is worth mentioning since it shows the best regenerative results following a TE uterus patch replacement to

date. In this study, the authors seeded the polymer based scaffold with endometrial and myometrial cells. Seeded constructs, and unseeded scaffolds, (6-8 cm × 2.5 cm) were transplanted to replace almost the entire uterus horn, leaving only a long strand of the native uterus for the attachment of the long U-shaped graft. Six months of follow-up showed that the seeded constructs had regenerated and that they contained cells positively stained for the uterus-specific cell markers estrogen receptor alpha and progesterone receptor. Pregnancy tests in grafted animals also demonstrated that the seeded constructs had normal pregnancies inside the graft site with the fetus body weights comparable to the controls. One major drawback of this study is that the authors failed to clearly demonstrate the site of embryo implantation and placentation, and it seems like these key events took place in the native (i.e. normal uterus) tissue that was kept during the TE engraftment. Another downside with this study is that the choice of using parametric statistical analysis for a sample size of 3 animals in each group.

Uterus tissue engineering in porcine models

In the only uterus TE study performed on the pig, to my knowledge, is Campo et al. from 2017.[109] The DC of the whole pig uterus was performed by vascular perfusion using an SDS and Triton X-100 protocol. Histology after DC showed no detectable cells while retaining essential ECM components such as collagen, elastin, fibronectin, laminin and GAGs. Quantifications of remnant DNA and protein content showed a marked reduction compared with the normal tissue control. The RC of 5mm punched biopsies with human stromal- and epithelial endometrial cells resulted in scaffolds rolling up into organoid like structures suggesting the possibility to create human like tissue using a scaffold from a xenogenic source.

Uterus tissue engineering in ovine models

There are two articles that have been published using the sheep animal model for uterus TE[110,231] and one manuscript submitted (Padma et al., under revision; Paper III herein). Daryabari et al., reported successful DC of whole sheep uterus using three protocols based on: (i) SDS and Triton X-100, (ii) DMSO and Triton X-100 and (iii) SDS and formalin. Histological analysis of the DC scaffolds revealed successful removal of DNA while simultaneously remained positive for collagen, elastin, fibronectin, laminin and GAGs (in all three protocols). Toxicity tests showed that scaffolds generated by all three DC protocols were non-toxic. Interestingly, the authors selected the DC scaffolds from SDS and formalin as being the best, and used these for a transplantation study into rats, despite it being a xenograft. The authors detected infiltrated cells on day 10 post transplantation. The cells within the grafts were stained positive for smooth muscle actin, cluster of differentiation (CD) 31, Ki-67 and vimentin and the authors conclude this as sings of functionality, angiogenesis, cell proliferation and fibro-connective tissue, respectively.

The other article on sheep uterus TE is Paper II[231] which together with a submitted manuscript (Paper III) is presented later in this thesis.

Uterus tissue engineering using human tissue

Two studies that investigated TE of human uterus tissue. In the first study, DC rat and human myometrium were produced using ethanol, water and trypsin.[232] These scaffolds were then RC with various rat and human monocyte cell lines where they cultured the constructs for up to 51 days in-vitro. Interestingly, the human cells adapted better to rat scaffolds where multicellular layers were formed within the depths of the scaffold. They also showed contractility in organ bath experiments, indicating a potential functionality. In the other study, the authors performed DC on 1-2cm^2 of endometrium layers of 0.5mm thickness using Triton X-100 and SDC with the aid of agitation.[179] They characterized the DC scaffolds and confirmed low DNA levels and a

retained ECM morphology. Additionally, the authors recellularized the scaffolds and cultured the constructs for up to 4 weeks, and then followed it up by a hormone cycle treatment for 28 days. The cells responded and were positive for both estrogen receptor and progesterone receptors indicating the presence of a uterus specific cell phenotype. With this small elaboration of the principles of TE and current advancements in the field of uterus bioengineering, the parts about my contribution to the field is mentioned from the next chapter.

Table 1. A summary of uterus TE studies reported until February 2021.
#The authors micro-dissected the endometrium and made into a hydrogel for downstream analysis.
¤The authors called infiltration of cells after transplantation of DC scaffolds as RC

Species	**Scaffold**	**DC**		**RC**						**Grafting**	**Pregnancy**	**Reference**
		Done?	Detergent	Done?	Cell source	Time	Culturing conditions	Done?	Time in-vivo	Tested?	When after Transplantation?	
Mouse	DC uterus	Yes	SDS	No	N/A	N/A	N/A	Yes	7 weeks	Yes	4 weeks	113
Rat	DC uterus	Yes	TritonX-100, DMSO, SDC	No	N/A	N/A	N/A	No	N/A	No	N/A	111,117
Rat	DC uterus	Yes	SDS	Yes	Rat neonatal, endometrial cells, MSCs	10 days	Bioreactor	Yes	90 days	Yes	28 days	116
Rat	DC uterus	Yes	SDS, TritonX-100, High hydrostatic pressure	No	N/A	N/A	N/A	Yes	51 days	Yes	30 days	118
Rat	DC uterus	Yes	TritonX-100, DMSO, SDC	Yes	GFP labelled MSCs	3 days	Static	Yes	9 weeks	Yes	6 weeks	112
Rat	DC uterus	Yes	SDS	No	N/A	N/A	N/A	Yes	11 weeks	Yes	8 weeks	115
Rabbit	#DC uterus	Yes	SDS, TritonX-199, DNase	No	N/A	N/A	N/A	N/A	N/A	Yes, in-vitro	N/A	230

Species	Scaffold	DC		RC						Grafting	Pregnancy	Reference
		Done?	Detergent	Done?	Cell source	Time	Culturing conditions	Done?	Time in-vivo	Tested?	When after Transplantation?	
Rabbit	Polymer scaffold	N/A	N/A	Yes	Myometrial, endometrial cells	4-5 days	Static	Yes	1, 3, 6 months	Yes	6 months	97
Pig	DC uterus	Yes	SDS, TritonX-100	Yes	Stromal, epithelial cells	3, 6, 9 days	Static but hypoxic	No	N/A	N/A	N/A	109
Sheep	DC uterus	Yes	SDS, TritonX-100, DMSO, Formalin	¤No	N/A	N/A	N/A	No	N/A	N/A	N/A	110
Sheep	DC uterus	Yes	SDS, SDC, TrironX-100, DNase	Yes	Sheep fetal MSCs	3, 14 days	Static	No	N/A	N/A	N/A	231
Sheep	DC uterus	Yes	SDS, SDC, TrironX-100, DNase	Yes	Sheep fetal MSCs	3, 14 days	Static, TW, MMP treatment	No	N/A	N/A	N/A	Paper III

AIM

The overall aims of the thesis were:

1. To investigate if the immune response towards decellularized uterus tissue was any different depending on what DC protocol was used in rats

2. To scale up the uterus decellularization process from the rat to the sheep model and establish a successful protocol for a large organ

3. To improve the recellularization efficiency of sheep uterus scaffolds

4. To optimize and establish conditions that can keep a uterus viable ex-vivo in a perfusion bioreactor that may benefit future whole uterus scaffold recellularization

MATERIALS AND METHODS

This chapter includes a brief description of the materials and methods used in the experiments for this thesis project. A more detailed description of the methods is outlined in each paper at the end of this thesis (Papers I-IV).

Study protocols for animal work (Papers I-IV)

The study protocols for Paper I and III followed the guidelines detailed in the animal ethics applications for rodents which was approved by the Animal Welfare committee at the University of Gothenburg, Sweden (114-2014 and 6878-2019). Papers II and IV did not require specifically approved animal ethics protocols since the sheep organs used came from a local abattoir for food production.

Animal work (Papers I-IV)

Rat uterus (Paper I)

For Paper I, 42 Female inbred Lewis rats and 3 outbred Sprague Dawley (SD) rats (Rattus norvegicus domestica) were used, all of which were 8-10 weeks old and between 140-180g in weight. Of these, twelve Lewis rats and three SD rats were used as donors. The remaining thirty Lewis rats were divided into five groups with six rats in each group. Group 1 received autologous grafts from their own uterus while group 2 received allogenic uterus grafts from SD rat donors. Groups 3, 4 and 5 received DC Lewis uterus tissue grafts that were developed from protocol (P) 1, P2 or P3 respectively.

Uterus procurement

The donor uteri used in Paper I for uterus scaffold production were isolated with an intact vasculature from the aorta to the vena cava to

enable whole organ DC through vascular perfusion. All vascular branches that did not lead to the uterus were ligated and cut. The specific uterus isolation procedures are detailed in an earlier publication and is summarized in Figure 5.[117]

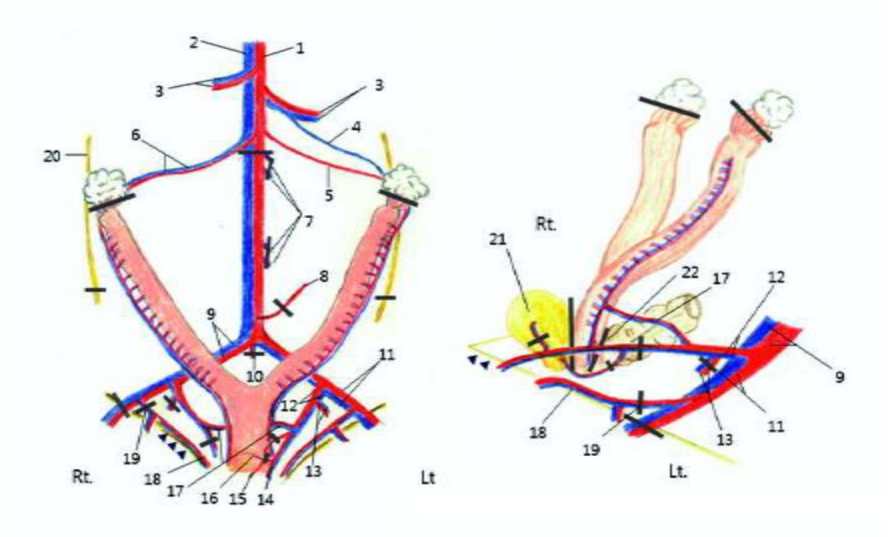

Figure 5. Figure used with permission from the publisher and authors.[117] A diagrammatic representation of the vascular anatomy of the rat uterus where the ligations are represented by black bars. 1=abdominal aorta; 2=vena cava; 3=renal vessels; 4= left renal vein; 5=left renal artery; 6=right renal vessels; 7=lumbar vessels; 8=inferior (caudal) mesenteric artery; 9=common iliac vessels; 10=caudal vessels; 11=external iliac vessels; 12=internal iliac vessels; 13=superior gluteal vessels; 14=umbilical vessels; 15=inferior vesical vessels; 16=superior vesical vessels; 17=uterine vessels; 18=external pudendal vessels; 19=inferior epigastric vessels; 20=ureter; 21=urinary bladder; 22=recto-sigmoid colon (Rt., right side; Lt. left side).

Rat embryonic dorsal root ganglion isolation (Paper III)

Two time-mated SD rats were deeply anesthetized and embryos (aged embryonic day 14) were collected and placed in L15 dissection media

and used for the dorsal root ganglion (DRG) assay elaborated later in this chapter.

Sheep uterus (Papers II-IV)

A total of 149 sheep uteri were isolated from female Swedish Finull or Texel breeds of sheep (Ovis aries) for Papers II, III and IV. More specifically, Papers II and III used a total of 121 sheep uteri isolated from 8-24 months old sheep. From these, 32 uteri of similar size were randomly selected for the following groups, with 8 uteri in each group: control, P1, P2 and P3. Meanwhile, Paper IV used 28 uteri from 9-12 months old sheep that were divided into four experimental groups, with each group receiving 7 uteri.

Uterus procurement

The uteri for Papers II, III and IV were obtained after a lethal trauma to the head that was followed by exsanguination according to standard culling protocols at the abattoir. Each uterus was dissected free from the surrounding tissue and cannulated through the uterine arteries. The cannulated uterus was then flushed until all the blood had been removed and until the specimen blanched using either ice-cold PBS supplemented with lidocaine and heparin (Papers II and III), or with ice-cold Institute George Lopez-1 (IGL-1) solution supplemented with lidocaine and heparin (Paper IV). Each uterus was then submerged in flushing solution and kept on ice during transport to the laboratory.

Decellularization

Rat uterus (Paper I)

The rat uterus DC procedure employed for Paper I has been elaborated extensively in earlier publications.[111,117] In short, P1 and P2 were DC with 4% DMSO and 1% Triton-X100 for 4h, respectively, followed by 16h of washing with PBS (P1) or deionized water (DW; P2). Protocol 3

was based on 2% SDC for 6h followed by 18h of washing with DW. The 24h cycles were repeated five times after which the scaffolds were sterilized by the perfusion of 0.1% per-acetic acid in normal saline for 30 minutes. Each uterus was then washed with sterile PBS and stored in -80°C until further used analyzed.

Sheep uterus (Papers II and III)

The DC process for the sheep uteri used in Papers II and III were performed in a custom-made perfusion DC setup. Briefly, P1 and P2 were perfused with 0.5% SDS or 2% SDC for 8h, respectively. This was then followed by the perfusion of DW for 16h. Protocol 3 was based on a combination of a 2% SDC solution (which was perfused for 4h followed by a washing step with DW for 8h) and a 1% Triton-X100 solution that was perfused for 12h. The procedure for P3 was repeated twice. The organs were then further perfused with DW to remove remnant detergents, this was followed by the perfusion of Dulbecco's PBS (DPBS) to saturate the tissue with a buffer to enable the perfusion of a DNase 1 solution (40IU/mL) at 37°C for 1h. After the enzymatic treatment, DW was perfused for 48h to wash the organ further. All the uteri were sterilized with the same method; the perfusion of 0.1% per-acetic acid for 1h. The sterilization liquid was then washed away with the perfusion of sterile PBS for 48h. The DC organs were then frozen at -80°C. Biopsies were taken for microscopy and quantitative analysis after thawing. For Paper II, the analysis of the DC sheep uterus tissue were conducted on cut ring sections with a thickness of 0.3-0.5cm. For Paper III, the DC uterus tissue was thawed and uterus scaffolds were made with a biopsy punch to create DC uterus tissue discs (dUTDs) 5mm in diameter comprising all the uterus layers (full-thickness of the uterus).

Bioreactor (Paper IV)

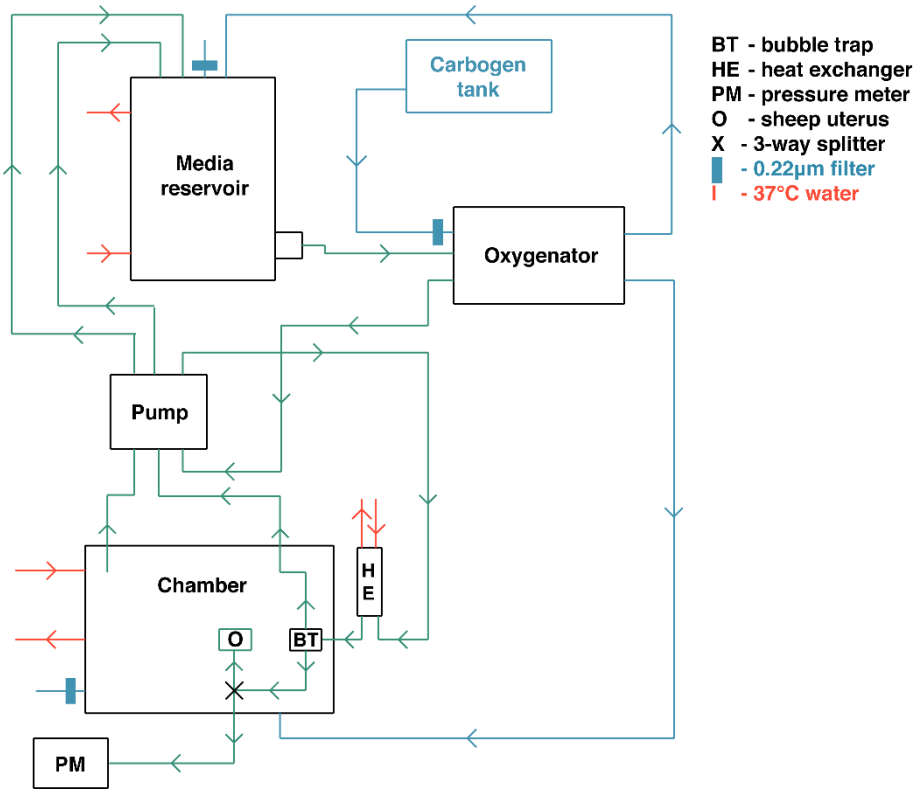

Figure 6. A schematic diagram of the perfusion bioreactor system used in Paper IV

For Paper IV, a customized cell culture medium was used as perfusion solution in the bioreactor. The perfusion medium composition had been optimized with different GFs and synthetic oxygen carriers individually, and then in various combinations using explants of sheep uterus rings. Two groups were used to study any cold ischemia induced detrimental effects on the uterus between the uterus isolation time and the normothermic reperfusion event started in the bioreactor (4h and 48h). The isolated sheep uteri exposed to the two alternative organ preservation times were connected to the Hugo Sachs electronics perfusion bioreactor system comprising a moist chamber, heat exchanger, oxygenator, media reservoir, pressure meter and a peristaltic pump (Figure 6). The media and all gas-filled spaces of the

bioreactor was enriched with carbogen gas (95% O_2 and 5% CO_2). A total of 250mL of recirculating perfusion medium for every organ was used and the arterial perfusion pressure maintained below 60mmHg (8kPa). Each organ perfusion group was compared to a control group where the uteri were instead kept in preservation solution at at +4°C. Biopsies and media samples were taken every 12h for histology and biochemical analysis, respectively.

Grafting surgery (Paper I)

The DC rat uteri used in Paper I were thawed from long-term storage, cut into 10×5mm pieces and sterilized with gamma irradiation. Three grafts per animal were transplanted subcutaneously to each rat under isoflurane anesthesia to assess a potential activated immune response caused by the grafts. The five experimental animal groups consisted of; 1) autologous grafts, 2) allografts, 3-5) sterile DC uterus grafts from each DC protocol, respectively. Each patch was secured to the underlying tissue with 6:0 polypropylene sutures and the wound was then closed using 4:0 silk sutures. The rats injected with an analgesic cocktail of carprofen and buprenorphine to reduce pain and inflammation. One biopsy from each grafted rat was retrieved at day 5, 15 and 30, respectively, and used for histological and gene expression analyses (see below) targeting recruited immune cells and pro-inflammatory cytokines.

Toxicity tests (Paper II)

For Paper II, a toxicity test was used to study if there were any cytotoxic remnant DC reagents in the scaffolds after the DC process using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay is based on the ability of metabolically active cells to convert MTT dye to the insoluble formazan crystals through the NAPD cycle. Any toxic compounds present in the culturing medium would affect the

metabolic activity of cells, and thus, toxicity correlates with lower formazan amount which is measured by spectrophotometry. In Paper II, the storage solution that the DC uteri had been kept in was included into the medium used to culture a standard cell culturing cell line (human embryonic kidney 293 cell cultures) along with the MTT dye.

Bioactivity tests (Paper III)

In Paper III, the functionality of remaining GFs in the scaffold after the DC process was evaluated using two different bioactivity tests:

The DRG assay: Isolated DRGs were placed on to either collagen coated wells or on the endometrial side of the dUTDs. The axonal regeneration of the cultured DRGs were assessed after two days and analyzed with immunohistochemistry.

The chicken chorioallontoic membrane (CAM) assay: The angiogenic activity of DC sheep uterus tissue was assessed using fertilized chicken eggs. A small window was opened in the shell of each egg on embryo development day (EDD) 3 and the shell opening was then closed using a patch of Tegaderm. On EDD 9, the shell window was reopened and an alginate hydrogel drop (control) or a DC uterus disc was inserted on the CAM (experimental groups represented each scaffold type). The egg was further incubated to EDD 14, and then the blood vessels that grew towards the insert were counted under an operating microscope to assess if the dUTDs simulated angiogenesis.

Mechanical tests (Paper II)

The mechanical strengths of native and DC sheep uteri were measured in Paper II since the physical properties of a scaffold used in bioengineering applications determine the fate of grafted cells, and have a major role in its degradation characteristics. For this, biopsies

were cut from the uterine horns and placed in a sheer stress modulus testing machine, where the force required to pull the tissues apart was measured. The maximum load (N/mm), tensile strength (J/mm), the amount of extension needed (%) and the sheer/stress modulus (Pa) were determined for each scaffold type and for the native uterus ring samples.

Fetal stem cell isolation (Papers II and III)

To isolate a cell population that could be advantageous for the RC of DC sheep uterus tissue and future sheep uterus bioengineering, heterogeneous sheep fetal bone marrow stem cells (SF-SCs) were isolated from 6-8 week old sheep fetus. The fetuses were obtained from pregnant sheep culled for food production. They were isolated from the uterus, dissected to expose their femur bones which were opened in both ends. The bone marrow was flushed out using Leibovitz L-15 medium. The bone marrow was collected in a tube and was cleaned by multiple centrifugation steps and was then plated out in a petri dish and cultured under standard cell culturing conditions. The cells were characterized and used for the RC procedures thoroughly explained in Papers II and III.

Recellularization (Papers II and III)

The DC sheep uterus tissue from all three protocols were recellularized in Papers II & III. More specifically, in both papers, the RC procedure was carried out in standard cell culturing conditions. However, further experiments were conducted in Paper III to improve the RC efficiency by using an enzymatic pre-treatment of the scaffolds before the cell seeding procedures. Additional improvements in RC were conducted by using TW inserts during cell culture (see below). All the RC scaffolds were analyzed using immunohistochemistry and microscopic analyses.

Recellularization under standard conditions

In Paper II, sectioned rings of DC sheep uterus tissues were recellularized with either 1×10^6 or 10×10^6 SF-SCs using a syringe and multiple sequential injections. Since there was no benefit using the higher concentration of cells, the lower concentration was used in Paper III. This study used the 5mm dUTDs (instead of the ring-shaped scaffolds used in Paper II) and was RC with the endometrial side up using the same cell application approach as in Paper II. The RC efficiency was assessed after standard cell culturing conditions for three days or for 14 days in-vitro.

Recellularization in transwells

In Paper III, with the intent to improve the RC efficiency by increasing the cell culture medium access to the myometrium side of the RC dUTDs (bottom), each construct was placed on a 0.4µm pore sized TW insert during cell culture. The dUTDs were recellularized with multiple injections the same way as mentioned above. The RC efficiency was again assessed after three days or 14 days in-vitro.

Recellularization after enzymatic preconditioning and transwell culture

An additional RC experimental group was included in Paper III. In this group, the dUTDs were enzymatically pre-treated for 24h with MMP 2 and 9 before being placed in the TW inserts, then they were recellularized and cultured as the other scaffold types before the RC efficiency was assessed.

Microscopic analyses (Papers I-IV)

In Papers I-IV, all the biopsies retrieved were fixed in either formaldehyde or glutaraldehyde for histology or electron microscopy, respectively. The biopsies were dehydrated and processed for the respective microscopic analysis mentioned below.

Histochemistry (Papers I-IV)

Hematoxylin and eosin (H&E) staining was performed in all Papers. Slides stained with H&E was also used to quantify the total number of cells per mm^2 (using ImageJ) in Paper I and for the microscopic measurements to quantify tissue edema in Paper IV. Additional standard staining protocols were used in Paper II to evaluate the morphology for ECM components before and after DC. These included Masson's trichrome (MT) stain, Verhoeff van Geison (VVG) stain and Alcian Blue (AB) stain to detect collagen, elastin and GAGs, respectively.

Immunohistochemistry (Papers I-IV)

To visualize DNA in processed tissue, sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) in Papers II, III and IV. Additionally, quantification of DAPI positive cells were used in Paper III to assess the RC efficiency, and was used in Paper IV to quantify tissue edema after normothermic bioreactor reperfusion of sheep uterus after they had been exposed to cold ischemia.

Bright-field immunohistochemistry of stained immune cells using antibodies for CD4 (T-cells), CD8a (cytotoxic T-cells), CD22 (B-cells), CD68 (macrophages), CD163 (activated macrophages) and natural cytotoxicity triggering receptor 1 (NK cells) Immuno-labelled positive cells were stained red with the Mach 3 kit and facilitated quantification of the specific cell types presented in the results for Paper I. Similarly, positive cells stained for cleaved caspase-3 were quantified in Paper IV to evaluate cold ischemia and reperfusion-induced apoptosis in the sheep uterus.

Immunofluorescent staining was performed in Paper II and III to characterize the phenotype of the SF-SCs used for the RC process, including the markers α-smooth muscle actin, vimentin, CD166, Ki67, estrogen receptor-α, estrogen receptor- β, progesterone receptor, cytokeratin, myoblast determination protein 1, receptor activator of

nuclear factor κ B and dentin matrix acidic phosphoprotein 1 before and after the RC procedure. Additional immunofluorescent staining was conducted in the DRG assay using a pan-neurofilament antibody to label regenerating axons.

Scanning electron microscopy (Papers II and III)

Scanning electron microscopy was performed on normal, DC and RC sheep uterus tissue and is presented in Papers II and III. To enable the visualization in the scanning electron microscope and to improve the contrast in content DC samples due to its low lipid content, each specimen was processed using the osmium tetroxide and thiocarbohydrazide (OTOTO) method.[233]

Biochemical analysis (Paper IV)

The components of the customized cell culture medium used as bioreactor perfusion solution for Paper IV were analyzed using a blood-gas system specifically measuring concentrations of sodium, potassium, glucose, pH and lactate. These factors provided relevant indicators for tissue/organ health during the bioreactor experiment.

DNA, RNA, protein and ECM quantification (Paper I and II)

Gene expression analysis was conducted in Paper I by isolating total RNA from tissue biopsies taken from the grafts at specific time-points after uterus scaffold transplantation. The RNA from these biopsies was then quantified by spectrophotometry, then converted to cDNA which was quantified in a droplet digital polymerase chain reaction (ddPCR; see below). In Paper II, DNA samples were isolated, quantified and loaded on an agarose gel to evaluate the size of remaining DNA after

the various DC protocols. This was conducted since large fragments of remaining DNA may affect the immunogenic properties of the scaffolds. Additionally, in Paper II, total protein and ECM components such as collagen, elastin and GAGs were isolated and quantified using optimized commercial colorimetric assay kits for respective components.

Gene expression analysis (Paper I)

All the procedures for ddPCR in Paper I followed the internationally established dMIQE guidelines for this type of work.[234] The isolated RNA was reverse transcribed to complimentary DNA using standard molecular kit-based methods after which the ddPCR were performed. In brief, each sample along with the master mix were divided into 16,000-20,000 individual droplets where the PCR reactions occur individually in each droplet. The PCR positive droplets get a “1” value and the negative droplets get a “0” value (hence, it is called digital) and the copies/µL was calculated using Poisson’s mathematics. The specific expression levels that were analyzed this way included the pro-inflammatory cytokines interferon-γ, interleukin (IL)-1β, IL-2, IL-6 and tumor necrosis factor.

Statistical analyses (Papers I-IV)

Statistical analysis for Papers I-IV were performed using Graphpad Prism. All data were tested for normal distribution with the Shapiro-Wilk test. When data sets were normally distributed, two group analysis was performed using the unpaired two-tailed Welch’s t-test, and multiple group analysis was performed using one-way ANOVA with Tukey’s HSD post-hoc analysis for multiple group corrections. For data with a skewed distribution (non-parametric), two group analysis was performed using the two-tailed Mann-Whitney U test, and multiple group analysis was performed with Kruskal-Wallis multiple group comparison corrected with Dunn’s post-hoc analysis. The data sets

were plotted either as bar graphs (mean ± standard error of mean), box plots (median, interquartile range and range) or line graphs (mean ± standard error of mean).

RESULTS

Paper I

In short, however, Paper I investigated if the process of DC affects the immunogenicity of the scaffold. ECM derived scaffolds should not provoke a strong immune response after transplantation since ECM molecules have a close molecular homology between species. However, the DC process and its chemicals may expose DAMPs that potentially could induce a detrimental immunological reaction after engraftment, even in a syngeneic transplantation setting. Hence, this needed to be investigated. Cells in biopsies isolated from transplanted syngeneic rats that received DC uterus tissue were quantified, and on day five after transplantation, the total infiltration of cells were significantly higher for P1 scaffolds compared with P2 and P3 scaffolds. The density of resident T-cells were significantly higher in P3 scaffolds compared with autologous grafts (control group). The cytokine expression levels of IL-1β was higher in P1 and P3 scaffolds, while IL-2 was elevated in P3 scaffolds. Fifteen days after transplantation, P3 grafts had an increased presence of cytotoxic T-cells, and the B-cell population had decreased in P1 and P2 scaffolds. The IL-1β expression remained higher in P3 derived scaffolds compared with the other groups. Thirty days after transplantation, there was no difference in any immune cell numbers that we analyzed, while IL-1β expression levels remained at a higher level in P3 scaffolds compared with the other grafts.

Overall, the immune response after the transplantation of the three different types of ECM derived uterus scaffolds had an early and a late immune response that were allogenic-independent. This suggests that DAMPs were formed during the DC process. The results indicated that the DC protocol that used mild detergent (P2) but was still effective in reducing the donor DNA content contained lower amounts of DAMPs and resulted in a moderate immune response, while an aggressive DC protocol (P3) seemed to have generated more DAMPs that triggered a stronger immune response.

Paper II

Macroscopic observations of the perfused sheep uterus revealed successful cannulation and organ procurement since the plastic replica model of the whole uterus vascular network were intact. The uteri blanched in color and turned white as the DC process progressed for all DC protocols (P1-P3). Microscopic analysis of the DC sheep uterus displayed the absence of cells (based on H&E stained slides), while the architecture of collagen, elastin and GAGs was retained (evident by the MT, VVG and AB staining, respectively). Scanning electron microscopy further demonstrated that the ECM ultrastructure contained intact collagen fibers and that it left a porous ECM structure with no visible cell structures present.

Quantification of the scaffold components presented a significant decrease of DNA content in all scaffolds compared with normal tissue. The remaining DNA fragment size were less than 500bp. The amount of total protein content and GAGs also decreased significantly compared with normal uterus tissue. Simultaneously, the amount of collagen decreased significantly in P1 and P3 scaffolds, while the elastin content significantly decreased in P2 and P3 scaffolds. Mechanical tests showed an increase for the maximum load in DC tissue after all three protocols, while the work required to destroy the samples remained unchanged in P1 and P2 scaffolds. The elasticity of the samples was increased in P1 scaffolds while P2 and P3 derived scaffolds remained closer in elasticity to the normal uterus.

All three DC scaffolds were found non-cytotoxic based on the MTT assay. There were viable cells near the injection sites in all RC scaffold types after 14 days in-vitro. However, it was obvious that the cells remained near the injection site and did not migrate much during the in-vitro period. This was irrespective of the DC protocol used. Interestingly, there was no difference in the RC efficiency when the constructs were seeded with 1 or 10 million cells. However, when taking all the results into consideration, P2 seemed to generate the best type of uterus scaffold compared with P1 or P3, since it had similar collagen content and mechanical properties to the native tissue.

Paper III

The experiments of Paper IV explored the scaffolds used in Paper II further and the RC strategies were further optimized. The study also looked into any potential functional bioactivity of the GFs in the DC scaffolds. More specifically, the DRG assay showed that dUTDs stimulated the DRGs to regenerate an increased amount of axons that grew longer distances compared with the control group, thereby demonstrating the existence of bioactive GFs that stimulated neurogenesis. In the CAM assay, the dUTDs resulted in a doubling of blood vessels in the vicinity of the scaffold, thereby demonstrating that the uterus scaffolds contained bioactive GFs that stimulated angiogenesis.

The RC of the scaffolds using standard culturing conditions showed that there was an increased ability for cell proliferation in P1 and P2 compared with P3 scaffolds. Using TW inserts during the cell culture increased the cell density in P1 and P2 scaffolds compared with P3 scaffolds short term, but did not seem to have a significant beneficial effect long term. Enzymatically pre-treated scaffolds that were cultured in TW inserts showed an increase in the RC efficiency in all three scaffolds, both in short and long term, compared with standard conditions. Interestingly, the pre-treatment of the scaffolds improved the RC efficiency even for P3 derived scaffolds which now became comparable with P1 and P2 scaffolds in cell density. In summary, pre-treatment of the DC scaffolds with MMPs prior to the RC process improved the RC efficiency and resulted in a 2 to 3 times increase in cell density compared with standard cell culturing conditions, independent of DC protocol used.

Paper IV

In paper IV, a perfusion bioreactor system was optimized and used for assessing cold ischemia and reperfusion damage of the normal sheep uterus. This experiment was conducted not only for its relevance to UTx

research, but also to optimize future whole sheep uterus scaffold RC experiments where such sophisticated 3D in-vitro culture system may prove beneficial.

Analysis of H&E stained slides revealed that the outer serosa layer and the inner columnar luminal epithelial cells was affected by edema during the normothermic reperfusion in the bioreactor, regardless of the duration of the cold ischemia. However, Group 1 with the shorter cold ischemia time did not show any increased edema in the endometrial layer while the uteri exposed to the longer ischemia time (Group 2) showed a significant swelling of this layer. This significant edema was also accompanied by an increased apoptosis that progressed with the duration of the reperfusion, something that was not seen in Group 1. The columnar epithelium in the lumen was affected in both groups as the normothermic reperfusion time progressed. However, this was a much more rapid process in the group previously exposed to the long cold ischemia time (Group 2) where the epithelium had completely disappeared after just 24h of reperfusion.

Biochemical analysis of the perfusate taken from regular intervals during the perfusion time revealed that the glucose concentration significantly decreased in later time points in both groups and that the pH significantly decreased over time only in Group 2. Lactate levels became elevated after 24h of uterus reperfusion in Groups 1 and after 36h in Group 2. The potassium levels were significantly increased in Group 2, but not in Group 1 that had the short initial cold ischemia time exposure. To sum up the general results for Paper IV, as expected, we saw a clear difference in organ quality depending on the exposure time of cold ischemia during the reperfusion event. The detailed differences observed using the reperfusion platform showed that the bioreactor may very well replace the need for large animal UTx experiments to study different cold ischemia and uterus preservation protocols to optimize UTx protocols. Additionally, this bioreactor system may similarly be used for uterus TE experiments where large uterus scaffold RC can take place in a controlled 3D in-vitro perfusion culturing system.

GENERAL DISCUSSION

Paper I

In Paper I, three different rat uterus scaffolds with a varying degree of remaining fragmented donor DNA were evaluated after transplantation in a syngeneic rat animal model. P1 scaffolds, which had the highest amount of donor DNA remnants as a result from the gentler DC protocol, caused a higher infiltration of cells during the first five days after engraftment. It is likely that this was caused by the higher presence of the nucleotide based DAMPs in this scaffold type compared with the other scaffolds evaluated. However, the total cell density in P1 grafts was reduced over time, suggesting a scaffold clearance from DMAPs and a potential path towards remodulation.[235]

Lymphocytes are normally recruited to the graft as a consequence of both the innate and the adaptive immune response. However, neither the density of $CD4^+$ T-helper cells nor the $CD8a^+$ cytotoxic T-cells were any different in the control groups or in P1 and P2 scaffold grafts, suggesting an absence of a negative immune response.[236,237] But the number of cytotoxic T-cells were higher in P3 grafts, indicating a potential presence of ECM-based DAMPs, which correlated with a higher $CD22^+$ B-cell population in this group 15 days post engraftment. Hence, the higher presence of both cytotoxic T-cells and B-cells in P3 may indicate that there was a progression towards rejection or unfavorable tissue regeneration in this graft type. Both T-cells and B-cells have shown to contribute to transplantation tolerance or mediate graft rejection by and induced cytotoxic response.[204,238] Additionally, macrophages can be involved in either rejection or wound healing depending on its activation and the proportions of the different macrophage phenotypes. The infiltrated $CD68^+$ pan macrophages and the infiltrated $CD163^+$ class M2 macrophages were not significantly different in any of the time points between the protocols. Although, transplantation of TE urinary bladder and small intestinal submucosa resulted in a higher anti-inflammatory (M2) macrophages,[239] we did not make similar observations. We also did not detect elevated levels of IL-

6, IFN-γ and TNF-α, that are related to higher pro-inflammatory (M1) macrophage presence.[240,241] M1 macrophages can stimulate angiogenesis through the M1-dependent VEGF signaling pathway and have been associated with degrading implants.[194] Hence, the low M2 macrophage density detected in this study may be due to a slow scaffold degradation. Additional studies would be need to validate these hypotheses, and further investigate the whole immunological response towards DC scaffolds. These studies should include anti-inflammatory signals (e.g. IL-1, IL-4, IL-10) on the protein level (serum analysis) and the mRNA level (ddPCR) using larger grafts transplanted orthotopically. To create the best possible scaffold, it is crucial to have an optimized uterus DC protocol that produces a minimal amount of DAMPs so that the scaffold can favor regeneration and tissue reconstruction. The clarification of optimal protocols to accomplish this will enable the translation of these bioengineering principles to larger animal models, and finally to the human.

In summary, the study of Paper I investigated the existence of allo-independent immunogenic DAMPs in uterus scaffolds generated from three DC protocols. We clearly demonstrated that one scaffold type (P2; produced by a mild DC protocol) did not seem to induce a detrimental immune response after engraftment.

Paper II

To scale up uterus TE protocols from a small to a large animal model is a challenging process, including choosing a relevant large animal model. Ribitsch et al. described in detail the advantages and disadvantages of using different animals for translational research.[242] For the uterus, the closest animal model to a human is a non-human primate model (e.g. macaque or the baboon model). However, it is difficult to obtain ethical approval for such experiments and it is also very expensive to work with these animals. Thus, we instead chose the sheep model. The sheep uterus has a close resemblance to the human uterus in terms of general size and morphology. Additionally, the sheep

rarely has more than two fetuses during pregnancy, while the pig model is considerably different to the human uterus.[243] However, there are some significant differences between the sheep and the human uterus that needs to be considered for translational work. For instance, invasive placentation that takes place during fetal implantation in humans is different to the sheep that instead is dependent on multiple smaller cotyledons-dependent placentae.[244] Additionally, the genetics of the sheep is often mixed to benefit food and fur production which consequently result in a relatively high heterogeneity between animals that can complicate research conclusions. However the sheep organs used in Papers II-IV came from a local slaughterhouse and provided us with a near unlimited supply of uteri that otherwise would have been discarded. In line with European animal welfare regulations, no requirement for an ethical permit was needed, thus, providing us with many advantages using this animal model. However, future transplantation studies will have to include exorbitant costs to cover housing and animal care during.

At the beginning of the sheep uterus DC study (Paper II), we conducted a pilot study to evaluate if the best protocol established on the rat (Paper I; P2; 1% Triton-X100 + 4% DMSO), could DC the sheep uterus. However, this protocol was found ineffective. Interestingly, this was contradicting to what Dayabari et al. concluded.[110] They used a similar protocol to P2 in Paper I, but they also used formalin in combination with this protocol for the sterilization process. The use of formalin is debatable, since, through its fixation process it severely impacts the ECM crosslinking. The consequences for this ECM crosslinking is not well documented and may result in unfavorable RC conditions and/or problematic outcomes following transplantation.

Our three different protocols for sheep uterus DC described in Paper II showed that there were protocol-dependent differences in scaffold composition with respect to collagen, GAGs, elastin amounts. The mechanical properties were also significantly affected by the choice of DC protocol used. Microscopic analysis and the quantifications

revealed that P2 that were based on SDC had a potential advantage over the other protocols.

The DNA quantities were minuscule in all three protocols suggesting that all these scaffold types should harbor a low immunogenicity caused by donor DNA. However, the GAGs content was also decreased in all protocols. This is a common consequence from the DC process caused by the combination of ionic detergents. GAGs are water soluble, thus making them even harder to maintain during perfusion-based DC protocols.[125] But this reduction could be overcome by supplementing or pre-treating the scaffolds with solubilized GAGs before any downstream RC process[245] which creates new ideas for future applications with the developed sheep uterus scaffolds.

Together with the MTT test, the developed uterus scaffolds' RC ability confirmed that the scaffolds were non-cytotoxic. Injected cells were predominantly found in the periphery of the uterine rings and were concentrated around the injection site. The observed limited distribution of cells after RC may be caused by a suboptimal cell injection procedure, and/or by a compact scaffold structure that negate cell migration. However, it may also be caused by remnant DC chemicals that was not properly extracted from the deeper tissue layers during the washing steps, plausibly causing a central cytotoxicity in some regions of the scaffolds. Better cell migration and a higher cell turnover should be beneficial for transplantation studies, hence, exploring this further and improve the RC efficiency would be of great value before future transplantations studies.

Overall, we observed that the rat uterus DC protocol needed to be modified in order to establish good sheep uterus scaffolds. Our results also showed that a uterus the size of a human uterus can be decellularized successfully. The developed P2 (the SDC-based protocol) seemed to be more favorable compared to the other evaluated protocols. Since this protocol also used a relatively mild detergent treatment, it may also contain less DAMPs, and thus, be more favorable after engraftment (Padma et al.; Paper I).

Paper III

The natural progression after establishing successful DC protocols of sheep uteri (Paper II) was to improve the RC strategies (Paper III). Efficient RC is not only dependent on how the cells are added to the scaffold and how the constructs then are cultured, but is also dependent on scaffold GF content. Since GFs support such an event, an abundance of studies quantifying different GFs in DC scaffolds were conducted.[111,230,246] However, the quantification methods used are based on denatured proteins. Thus, the results from these studies do not demonstrate GF functionality. Functional GFs are crucial to facilitate the seeded stem cell migration and proliferation, and to facilitate reconstruction after implantation and angiogenesis.[247]

Two bioactivity tests were performed in Paper III to assess scaffold bioactivity and the presence of functional GFs: the DRG and the CAM assays (neurogenesis and angiogenesis, respectively). It was evident from the DRG assay that all three dUTDs induced axonal growth and that the axons grew longer compared with the collagen substrate control group. This indicated that there was a presence of neurogenic GFs. This method however, did not discriminate specific GFs responsible for different parts of neuronal sprouting.[248] There are cytokines such as IL-6 that are responsible for tissue regeneration, including for uterus tissue,[113] which also are known to play a role in growth of DRGs.[154] Nonetheless, the exact mechanism needs to be further investigated in order to improve regeneration post implantation of the constructs. The CAM assay confirmed angiogenic properties of the dUTDs. More specifically, a near doubling of blood vessels were formed around the dUTDs compared with the control group. Angiogenic GF remnants in the scaffolds could be advantageous after engraftment as it would reduce the ischemia after transplantation. However, additional studies need to be done in order to determine if the effects are caused by functional GFs or by inherent properties of the scaffolds. This is an interesting area for further research that could include setting up similar assays but with the addition of specific GF inhibitors in the cell culture medium to determine growth-dependent signaling pathways (e.g. VEGF, STAT3 or IL6).[113,154]

Recellularization is a demanding feat and few studies have actually shown high cell densities in ECM scaffolds that were quantified in an unbiased manner and compared with native tissue. Furthermore, many studies used constructs that were grafted after a relatively short time in-vitro after the RC process. Additional heterogeneity between TE studies include method for cell delivery,[181] time cultured in-vitro after RC[249] and by what type of construct culture system used (e.g. static cell culture, a bioreactor system or culture spinner flasks).[184,250] The choice of cell type used for the RC also plays an important role. These variables make studies difficult to compare. We used heterogeneous multipotent SF-SCs for our RC experiments in Paper II and III. They demonstrated multipotency before and after RC, thereby indicating that the cells did not differentiate in-vitro when cultured with the sheep uterus scaffolds. Similar cell types have been established to produce GFs and ECM components that are especially responsible for tissue regeneration,[251] activate repair mechanisms,[218] and a similar cell type have shown to play a beneficial role in endometrial repair following cell transplantation to the uterine spiral arteries in a clinical setting.[252]

The previous study (Paper II), and most other studies commonly use the strategy described as “standard culturing condition” in Paper III. As observed in other studies, this method resulted in a limited RC efficiency with a sparse cell aggregation concentrated around the injection sites and not clear indications of cell migration towards the deeper scaffold structures.[109,112,231] When the scaffolds were RC and cultured on TWs, the cell density improved compared with standard culturing conditions. The cells also migrated further from the injection site which are similar observations to what was documented in another study on kidney RC.[147]

The physical properties such as porosity of the scaffolds can affect the RC efficiency.[253,254] With this in mind, we pre-treated the scaffolds with MMP-2 and MMP-9 that are widely expressed by proliferative and migrating cells that use these enzymes to breakdown the ECM components to ease their mobility[251,255] and accelerate angiogenesis.[256] When the scaffolds were treated with MMPs and then RC and cultured

in TWs, there was a considerable advantage seen already on day 3, and an even more pronounced benefit noted after 14 days in culture. Even the least efficient scaffold (P3) became comparable to the other two scaffold types with regards to cell density and migration. The cells clearly migrated better and proliferated more in the scaffolds after the MMP treatment irrespective of the DC protocol used for scaffold generation.

In essence, this study displayed bioactivity in DC uterus scaffolds that stimulated neurogenesis and angiogenesis. The MMP preconditioning of scaffolds before RC and TW culture improved the cell density by 200%-300% compared with standard culture conditions, irrespective of DC protocol used for scaffold production. Additional studies may include optimizing the cell seeding procedure further, e.g. using circular multi-needle injectors instead of repeated single injections, prolonged in-vitro periods for RC constructs (e.g. 1-2 months) and the RC of larger scaffolds. Furthermore, the administration and efficacy of the MMP preconditioning treatment needs to be tested on whole organ RC to evaluate if this can potentially be combined with the use of perfusion bioreactors for successful RC of large constructs and future transplantation studies on the sheep uterus.

Paper IV

An extracorporeal perfusion bioreactor is commonly used to preserve or recondition an organ before organ transplantation in the clinical setting.[182,257] This is usually conducted below 4°C. However, Recent organ preservation studies indicated that a normothermic extracorporeal organ perfusion setup using a blood based perfustate exhibited milder organ damage after transplantation compared with standard cold storage procedures for the liver[258] and kidney.[257]

Similar strategies might be used for whole organ RC applications in TE. However, whole organ RC in a perfusion bioreactor is challenging and requires constant monitoring of a multitude of variables, including

medium composition (e.g. glucose, pH, lactate and osmolality), oxygen saturation, vascular pressure and edema prevention.

In order to establish some base line criteria and facilitate for future whole organ RC experiments on the sheep uterus scaffolds created in Paper II, we designed a normothermic perfusion platform for the sheep uterus in Paper IV. This perfusion platform was also relevant for studying uterus ischemia and reperfusion induced injury, which is still not a well characterized area. In the clinic, the uterus is not an essential organ for transplantation. Therefore, it is likely that other organs will have priority from a multi-organ donor during procurement. This may lead to longer cold ischemia times compared with the easier logistically planned living donor procedures that can allow shorter ischemia times. For example, the cold ischemia time for the first successful UTx that used a deceased donor was 6.5h[69] while the cold ischemia time for the succesful live donor UTx cases was about 1.5h.[259]

Our perfusion system used a peristaltic pump to circulate the perfusate. Since this can cause hemolysis of red blood cells,[260,261] we developed a perfusion solution containing perfluorocarbons as synthetic oxygen carriers to substitute the red blood cells. These oxygen carriers have successfully been used in the clinic intravenously to elevate saturated oxygen levels in the blood[190] and reduced cerebral ischemic damage and demonstrated non-toxicity.[191] We also added reactive oxygen species scavengers to counteract potential detrimental effects from reactive oxygen species released during the reperfusion.[85]

Many clinical and pre-clinical studies on organ preservation use standard solutions like Perfadex, Histidine-Tryptophan-Ketoglutarate, University of Wisconsin, IGL-1 or Steen solution, all of which contain high levels of either polyethylene glycol, mannitol or albumin to reduce edema formation. These also contain low (or no glucose) to decrease the metabolic activity in cold temperature perfusion systems. As a result, the lactate levels are respectively low under such settings. However, in Paper IV, the perfusion was performed at normothermic temperatures (37°C). Therefore, when the perfusate was analyzed after the perfusion of the sheep uterus at different time-points, the lactate

levels had increased while the pH decreased. These parameters are explained by the recirculation of the perfusion medium for 48h and the high metabolic activity in the organ. These metabolites may be detrimental for the organ and better results may have been obtained if the perfusion medium was not recirculated, or if the media was replaced at regular intervals.

However, when the organs were histologically analyzed, a low degree of apoptosis was seen in the group exposed to a short ischemia time. These are good results considering that we performed the normothermic perfusion for 48h in the bioreactor. We observed more cleaved caspase-3 positive apoptotic cells in the group exposed to 48h of cold ischemia. This ischemic-related damage was expected since such duration is considerably long, and far from optimal for organ transplantation.

The system may be optimized further, for example if the maintenance of the osmolality during the perfusion was kept at about 300mOsm/L.[262] The luminal epithelial layer in both groups degraded during the perfusion which may have been caused by changes in osmolality during the experiment.[263] Additionally, the edema observed after prolonged reperfusion in both groups could potentially be dampened by increasing the albumin or polyethylene glycol concentrations and/or lower the solute concentration.

From a UTx perspective, we were able to discriminate differences in cold ischemia-related injuries using this developed normothermic perfusion system, and we demonstrated that these bioreactor settings could act as a substitute for expensive in-vivo experiments to assess the reperfusion injury. Hence, this system could be used to evaluate different types of cold preservation solutions for uterus procurement at a much lower cost and effort than using live animals for transplantation studies. Additionally, we established base line criteria for the medium composition and perfusion bioreactor settings. These important parameters will assist us to develop whole organ RC protocols for future uterus bioengineering applications on large organs.

CONCLUSIONS

Paper I — The immunogenicity of the scaffolds was DC protocol dependent, and the remaining donor DNA also played an important role. The rat uterus scaffolds generated from P2 that used a milder decellularization protocol contained fewer DAMPs and was better than the scaffolds produced by a more aggressive detergent. Therefore, P2 scaffolds should be the focus for future uterus TE studies on the rat model.

Paper II — To scale up DC protocols from the rat to the sheep model warrants the establishment of new DC protocols. The sheep uterus scaffolds produced by an SDC-based protocol seem to be the best among the developed protocols as it retained the ECM morphology better and resulted in scaffolds with comparable mechanical strength to the native sheep uterus.

Paper III — All the decellularized sheep uterus had functional bioactive GF that stimulated neurogenesis and angiogenesis. Preconditioning of the scaffolds prior to RC using MMPs, and culturing the constructs in TW inserts increased cell density by 200-300% compared with normal RC and culturing strategies, independent of protocol used for decellularization.

Paper IV — We developed a perfusion bioreactor system that could maintain sheep uteri viable ex-vivo for 48h where cold ischemia-related injuries could be assessed after reperfusion. Hence this platform could be used to assess different novel preservation solutions for uterus tissue to improve UTx protocols. These perfusion parameters also serve as an excellent baseline to develop novel RC protocols for whole sheep uterus bioengineering.

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